IDENTIFICATION AND INVESTIGATION OF CANDIDATE TUMOUR SUPPRESSOR GENES FROM CHROMOSOME 11q24-q25 IN EPITHELIAL OVARIAN CANCER

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2002

I declare that this thesis has been composed entirely by myself and that this is my own work except where I have indicated the contribution of others.

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July 2002

ACKNOWLEDGEMENTS

I am grateful to Scottish Hospitals Endowment Research Fund and Edinburgh University Faculty of Medicine for funding me to do this research. I would particularly like to thank Hani Gabra for his support and encouragement throughout this period. I would also like to thank Grant Sellar for teaching me many of the techniques that I have learnt over the past three years. I would like to thank the scientific officers, Eric Miller and Diane Scott who performed most of the SSCPE and Gen Rabiasz who performed the methylation specific PCR. Venturing into an unknown world in the lab from the safe haven of clinical work would have been a far more daunting prospect had it not been for the help and friendliness of everybody in the lab.

July 2002

ABSTRACT

Approximately 7000 women are diagnosed with epithelial ovarian cancer every year in the UK. The majority of these women will die from their disease. Understanding the molecular biology of epithelial ovarian cancer will lead ultimately to the identification of targets for therapeutic intervention and diagnosis.

Tumour suppressor genes play a crucial role in carcinogenesis yet few of these genes have been identified in sporadic ovarian cancer.

Previously published studies suggest that a tumour suppressor gene in epithelial ovarian cancer is located at chromosome 11q24-5. To identify the tumour suppressor gene from this region I have performed the largest LOH study in this region to date using 16 polymorphic microsatellite markers and DNA pairs from 112 patients with epithelial ovarian cancer. The results from this study have led to the identification of several critical regions of LOH. One of these regions unambiguously identifies OBCAM as a strong candidate tumour suppressor gene.

OBCAM is an extracellular GPI-linked cell adhesion molecule that is shown in this work to be almost ubiquitously inactivated in epithelial ovarian cancer by a combination of LOH and methylation. Furthermore I have demonstrated that it is a functional tumour suppressor both *invitro* and *invivo*.

OBCAM is the first member of the IgLON family of proteins identified as likely to be important in cancer.

LIST OF ABBREVIATIONS

APLP2	Amyloid precursor protein 2
APC	Adenomatous polyposis coli gene
BAC	Bacterial artificial chromosome
BLAST	Basic local alignment search tool
bp	Base pairs
BT	Bos taurus
CA-125	Ovarian cancer antigen CA-125
CAM	Cell adhesion molecule
CDH1-12	Cadherin 1-12
CE	Caenorhabditis elegans
CCAP	Cancer Chromosome Aberration Project
CGAP	Cancer Genome Anatomy Project
CHK1	Checkpoint 1 gene
DCC	Deleted in colorectal cancer gene
DEPC	Diethylpyrocarbonate
DHPLC	Denaturing high pressure liquid chromatography
DM	Drosophila melanogaster
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide phosphate
ECM	Extra cellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

EOC	Epithelial ovarian cancer
EST	Expressed sequence tag
FCS	Fetal calf serum
FIGO	International Federation of Obstetricians and Gynaecologists
FISH	Fluorescent in-situ hybridisation
g	Grams
GG	Gallus gallus
HET	Heterozygous, no loss
HOSE	Human ovarian surface epithelium
HS	Homo sapiens
Ig	Immunoglobulin
IgLON	Immunoglobulin subfamily of OBCAM, LAMP and NTM
IP	Intraperitoneal
Kb	Kilobases
LAMP	Limbic system associated membrane protein
LOH	Loss of heterozygosity
M	Molar
µM	Micromolar
Mb	Megabases
Min	Minutes
ml	Millilitres
MRC HGU	Medical Research Council Human Genetics Unit
mRNA	messenger RNA

MSP	Methylation specific polymerase chain reaction
NCBI	National Center for Biotechnologies Information
NIX	Nucleotide Identify X
NF1	Neurofibromatosis gene 1
NF2	Neurifibromatosis gene 2
NTM	Neurotrimin
OBCAM	Opioid Binding Cell Adhesion Molecule
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RB	Retinoblastoma gene
RNAse	Ribonuclease
RNA	Ribonucleic acid
RN	Rattus norvegicus
Rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SC	Subcutaneous
Secs	Seconds
SRO	Shortest Region of Overlap
SSCPE	Single Strand Conformation Polymorphism Electrophoresis
SKY	Spectral karyotyping
STS	Sequence tagged site
TSG	Tumour suppressor gene

VHL	Von Hippel-Lindau gene
WT1	Wilm's tumour gene

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1. INTRODUCTION

1.1 EPITHELIAL OVARIAN CANCER

1.1.1 Epidemiology

Ovarian cancer is the 4th most common cancer among women in the United Kingdom after breast, lung and colon cancer respectively. In 1997, there were 6820 new cases of ovarian cancer in the UK accounting for 5% of the cancer burden in women. The lifetime risk of developing ovarian cancer is approximately 1 in 48 and it is predominantly a disease of postmenopausal women. The incidence of ovarian cancer varies among different geographic regions and ethnic groups with a high incidence in northern Europe and the United States and a low incidence in Japan.

1.1.2 Aetiology

The majority of ovarian cancers are sporadic suggesting that almost all cases can be attributed to spontaneous or environmentally induced carcinogenesis. Whilst there are several models of ovarian carcinogenesis the aetiology of ovarian cancer is poorly understood. In his theory of incessant ovulation Fathalla proposed that chronic repeated ovulation without pregnancy contributes to neoplasia of the surface epithelium (Fathalla 1971b). Many observations support this theory. A further hypothesis suggests that ovarian neoplasia is a result of excessive hormonal stimulation. The ovary is responsive to steroid and gonadotrophin hormones and perturbations of these hormone levels may lead to excessive ovarian epithelial proliferation and malignant transformation. Exposure of the ovaries to pelvic contaminants and carcinogens may play a role in carcinogenesis and lifestyle factors such as dietary and alcohol intake and cigarette use have been

investigated. The most influential risk factors for ovarian cancer are age and a family history of the disease.

1.1.2.1 Incessant Ovulation

The necessary proliferation of the ovarian surface epithelium after ovulation suggests that the process of ovulation may play a role in the aetiology of ovarian cancer. Poultry hens that have been kept hyperovulatory for long periods have a tendency to develop ovarian or tubal adenocarcinomas (Wilson 1958).

Factors such as pregnancy that suppress ovulation are associated with a reduction in risk of ovarian cancer. Many epidemiological studies have found parity to be protective against ovarian cancer (Risch, Marrett, and Howe 1994) (Modan et al. 2001). Multiparas have a risk reduction of between 40 and 60% as compared to nulliparas. It has been estimated that each successive delivery confers a 16 to 22% risk reduction (Risch 1997).

Use of the oral contraceptive pill is associated with reduced risk of ovarian cancer. In a 25 year follow up cohort of 46000 British women, amongst current and recent users (within 10 years) the relative risk of death from ovarian cancer was 0.2 (Beral et al. 1999). By increasing the number of ovulatory cycles in a woman's lifetime it has been suggested that early menarche and late menopause may be associated with an increased risk of ovarian cancer. Breast-feeding can suppress ovulation and as such may be expected to offer some protection from ovarian cancer (Siskind et al. 1997).

1.1.2.2 Hormonal factors

Excessive gonadotrophin stimulation is postulated to be a risk factor for ovarian cancer. This hypothesis was first formulated after observations that ovarian tumours occurred in rodents following bilateral oophorectomy and ovary transplantation under the splenic capsule (Biskind 1944). The same author subsequently demonstrated that intact ovarian function in these rodents suppressed tumour formation apparently by reducing the gonadotrophin hypersecretion (Biskind 1948). However, in a prospective study, Helzlsouer et al (Helzlsouer et al. 1995) found that case subjects with ovarian cancer had significantly lower levels of FSH than control subjects. There was no difference in serum LH levels. Overall the evidence suggests that whilst gonadotrophins are involved in feedback regulation of ovarian steroid hormone production they in themselves may not be responsible for alterations in ovarian cancer risk.

1.1.2.3 Estrogens

During menstrual cycles, the ovarian surface epithelium proliferates at times when estrogenic influences are high. At these times, mitotic activity is high and thus the epithelium is vulnerable to acquiring mutations. As well as its role in inhibiting ovulation, the protective effect provided by the contraceptive pill may in part be due to reduction in endogenous estradiol production through suppression of the midcycle LH peak.

Evidence suggesting that estrogen is not a risk factor derives from the knowledge that pregnancy is protective and yet is associated with dramatically elevated serum estrogen

levels. Contradictory evidence has been accumulated on the effect of postmenopausal estrogen replacement therapy and ovarian cancer risk.(Kaufman et al. 1989; Risch 1996) (Purdie et al. 1995b). The most recent study published concludes that ever users of estrogen only hormone replacement therapy and users of hormone replacement involving administration of sequentially added progestins may be at increased risk of epithelial ovarian cancer (Riman et al. 2002). Users of continuously administered progestin hormone replacement therapy were not found to be at increased risk.

Normal ovarian surface epithelial cells and ovarian tumours have been shown to express estrogen receptors although only a small number of tumours respond to anti-estrogen therapy (Brandenberger, Tee, and Jaffe 1998). A study of 35 normal ovaries using monoclonal antibodies was barely able to detect estrogen receptors in surface epithelial cells or in inclusion cysts (Zeimet et al. 1994). The same study showed progesterone receptors in 85% of sections of surface epithelium and 100% epithelial inclusion cysts.

1.1.2.4 Progestogens

The normal ovarian surface epithelium is capable of both estradiol and progesterone production. In a study by Ivarsson et al, progesterone appeared to act as a negative regulator of ovarian surface epithelial growth while estradiol had no effect (Ivarsson et al. 2001).

The elevated levels during pregnancy suggest a possible protective role of progesterone in the aetiology of ovarian cancer. The progestogens in oral contraceptive pills may contribute to the reduced risk of ovarian cancer in users by a mechanism other than suppression of ovulation. Progestogen only pills do not totally suppress ovulation and

ovulatory cycles do occur. In a case control study of women who had used progestogen only contraceptive there was a significant reduction in ovarian cancer risk compared to never users. Use of depot medroxyprogesterone acetate (DMPA), a progestogen that suppresses endogenous progesterone synthesis and ovulation does not appear to affect the risk of epithelial ovarian cancer (Kaunitz 1996).

Physical activity (without amenorrhoea or other menstrual disturbances) is associated with decreased progesterone levels (Ellison and Lager 1986). In a study of 31000 Iowa women who were followed for greater than 7 years there was an increased risk of ovarian cancer with increasing levels of physical activity (Mink et al. 1996).

1.1.2.5 Androgens

The presence of androgen receptors in the ovarian surface epithelium has been directly demonstrated (al-Timimi, Buckley, and Fox 1985) and the ovarian surface epithelium has been demonstrated to be an androgen responsive tissue (Edmondson, Monaghan, and Davies 2002).The function of androgens within ovarian epithelial cells is unclear. In the ovaries the secretion rate of androgens is greater than that of oestrogens. Androgens are present in follicular fluid and are the principal sex steroid of fluid in growing follicles (McNatty et al. 1979). There is evidence to support the relationship between androgens and the risk of ovarian cancer. Helzlsouer et al showed that as well as normal LH and slightly lower FSH levels than controls, patients with epithelial ovarian cancer had significantly elevated levels of androgens (Helzlsouer et al. 1995). A further piece of evidence concerns the association observed between history of polycystic ovary syndrome and risk of epithelial ovarian cancer. In general these patients have elevated

serum androgen levels and in the Cancer and steroid hormone case-control study, case subjects were more likely to have been diagnosed with polycystic ovary syndrome than controls (Schildkraut et al. 1996).

1.1.2.6 Environmental and dietary factors

Perineal talc exposure has long been debated as a risk factor for the development of epithelial ovarian cancer. There have been many epidemiologic studies providing conflicting results (Cramer et al. 1999) (Purdie et al. 1995a; Wong et al. 1999). Tubal sterilisation and hysterectomy have both been demonstrated to provide a reduction in risk of ovarian cancer (Green et al. 1997). An explanation for this finding is that contaminants from the vagina such as talc gain access to the peritoneal cavity through patent fallopian tubes and may enhance or promote malignant transformation of the ovarian surface epithelium either directly or as a consequence of inflammation. Surgical tubal occlusion may reduce risk by preventing the access of such agents.

Evidence of dietary risk factors for ovarian cancer is inconsistent. Several studies have found an increased risk of ovarian cancer with saturated fat intake (Risch et al. 1994) but others have not found the same relationship (Kushi et al. 1999). Moderately elevated risks have been found with lactose and cholesterol intake and total vegetable intake was modestly associated with reduced risk of ovarian cancer (Risch et al. 1994) (Verhoeven et al. 1996). Analysis of risk associated with coffee, alcohol and tobacco intake has also been investigated but findings have generally been inconclusive (Kato, Tominaga, and Terao 1989) (La Vecchia et al. 1992) (Marchbanks et al. 2000; Polychronopoulou et al. 1993) (Kuper et al. 2000).

1.2 EPITHELIAL OVARIAN CANCER: BIOLOGY AND PATHOPHYSIOLOGY

1.2.1 Embryological development of the ovary and female reproductive system

Knowledge of the embryological development of the female reproductive system provides a basis for understanding the range of tumour types that can develop from the ovary.

During the third week after fertilisation, gastrulation occurs. Gastrulation is the process that establishes the three germ layers in the embryo (i.e. the ectoderm, mesoderm and endoderm). The mesoderm develops to be comprised of paraxial mesoderm, intermediate mesoderm and lateral plate mesoderm. The ovary and indeed the rest of the urogenital system develop from the intermediate mesoderm (Sadler 1990).

The sex of the embryo is genetically determined at the time of fertilisation, however the gonads do not acquire male or female morphological characteristics until the seventh week of development. The gonads initially appear as a pair of longitudinal ridges called the genital ridges. These ridges are formed by proliferation of the coelomic epithelium and a condensation of the underlying mesenchyme, both of which are derived from the mesoderm layer. The germ cells appear in the wall of the yolk sac and migrate to the region of the genital ridge by approximately four weeks. Concurrently, the coelomic epithelium overlying the ridge begins to proliferate and forms primitive sex cords, which penetrate into the underlying mesenchyme. At this stage of development it is impossible to differentiate between a male and female gonad and it is therefore known as the indifferent gonad. In the absence of a Y chromosome, the gonad develops into an ovary. The primitive sex cords degenerate and are replaced by a second generation of cords

called cortical cords. The cortical cords then degenerate into cell clusters surrounding the germ cells. These surrounding cells develop into follicular cells and the germ cells develop into oogonia.

The reproductive system develops in close association with the urinary system, both being derived from the mesoderm. Initially in the embryo there are two pairs of genital ducts namely the mesonephric (or Wolffian ducts) and the paramesonephric (or Mullerian ducts). In the female the mesonephric ducts degenerate whilst the paramesonephric ducts continue to develop. The paramesonephric ducts arise as a longitudinal invagination of the coelomic epithelium on the anterolateral aspect of the genital ridge. Cranially the ducts open into the coelomic cavity and ultimately develop into the Fallopian tubes. Caudally the two paramesonephric ducts lie adjacent to each other and eventually fuse to form the uterine canal which develops into the body of the uterus, cervix and upper one third of the vagina. The lower portion of the vagina is formed from the urogenital sinus.

1.2.2 Neoplasms of the ovary

The vast majority (85%) of ovarian tumours arise from the surface epithelium of the ovary (Auersperg et al. 2001). The remainder arise from germ or stromal cells and will not be discussed further.

In the embryo the coelomic epithelium gives rise to both the ovarian surface epithelium and the Mullerian duct system. It can therefore be appreciated that when the ovarian surface epithelium becomes malignant it can express a variety of Mullerian type differentiations i.e. serous (akin to fallopian tube epithelium), endometrioid (akin to the

uterine endometrium) and mucinous (akin to the cervical epithelium). Tumours of the ovary can be benign, borderline or malignant.

1.2.2.1 Benign tumours

Benign tumours grow by expansion often compressing adjacent tissues and developing a rim of condensed connective tissue at their periphery. Unlike malignant tumours, benign tumours are not invasive or infiltrating and they have an excellent prognosis. Histologically, cells within most benign tumours closely mimic corresponding normal cells. Benign tumours do not have the capacity for metastasis.

The most common benign tumour derived from the ovarian surface epithelium is the serous cystadenoma. These tumours present as large spherical masses and are bilateral in 20% of cases. Macroscopically these tumours are usually unilocular cysts that have a smooth inner lining. Microscopically the appearance resembles that of the fallopian tube.

Benign mucinous tumours tend to be larger than their serous counterparts. They are often multiloculated and filled with thick mucinous material. Approximately 5% of these tumours are bilateral. Benign endometrioid tumours are rare. Transitional cell or Brenners tumours are thought to arise through metaplasia of the ovarian surface epithelium to form urothelial nests. They are normally unilateral.

1.2.2.2 Borderline tumours

Borderline tumours or tumours of borderline / low malignant potential account for approximately 4 – 14% of all ovarian malignancies (De-Vita, Hellman, and Rosenberg 1997). The presence of epithelial budding, multilayering of the epithelium, increased

mitotic activity and nuclear atypia distinguish these tumours from benign mucinous and serous tumours. However, like their benign counterparts they do not breach the basement membrane and there is no stromal invasion. Borderline tumours can however locoregionally disseminate. These tumours have a relatively benign prognosis. The tumours can be large and mucinous tumours can be associated with pseudomyxoma peritonei. In this condition, the presence of large quantities of mucinous material in the abdominal cavity is often fatal. As well as mucinous and serous tumours, Brenner and endometrioid borderline tumours have been reported but are rare.

Borderline tumours should be staged in the same manner as malignant tumours. Surgery is the main treatment for this disease. There is little evidence to suggest that treatment beyond that of initial surgery has any beneficial role. This is thought to be the same for both stage II and III disease as well as stage I disease.

1.2.2.3 Malignant tumours

Malignant tumours tend to grow faster than benign tumours. Histologically they display patterns ranging from well differentiated to poorly differentiated or anaplastic. Anaplastic cells are characterised by nuclear and cellular pleomorphism. Malignant tumours demonstrate an elevated nuclear/cytoplasmic ratio and abundant mitoses. Malignant tumours are invasive and infiltrating and can destroy normal surrounding tissues. Invasion is identified histologically as breach of the basement membrane. Finally, malignant tumours are distinguished by their ability to metastasise to sites discontinuous with that of the primary tumour.

Serous tumours are the most common malignant tumour arising from the ovarian surface epithelium. Two thirds are bilateral. Macroscopically, serous cystadenocarcinomas often have small mural papillary projections. Histologically they are composed of a multilayered epithelium with many papillary projections forming solid epithelial masses.

Malignant mucinous tumours form multilocular cysts and are bilateral in approximately one fifth of cases. Seeding of the peritoneal cavity with multiple implants can result in pseudomyxoma peritonei.

Endometrioid tumours are distinguished by their resemblance to glands of the endometrium. One third of cases have coexisting endometriosis.

Clear cell carcinoma of the ovary frequently presents with early stage disease as a large pelvic mass. There is an association with endometriosis, vascular thrombotic events and hypercalcaemia. Clear cell tumours tend to respond poorly to platinum based chemotherapy and their overall prognosis is poor.

1.3 EPITHELIAL OVARIAN CANCER: CLINICAL MANAGEMENT AND PREVENTION

1.3.1 Prognostic factors

Many different clinical, biological, pathological and surgical factors have been evaluated as predictors of outcome in ovarian cancer. The importance of some of these prognostic factors is undisputed but for others the evidence is not so clear.

Tumour stage at the time of diagnosis is undoubtedly one of the most significant prognostic factors. The SEER study in 1999 reported five year survival rates for ovarian

cancer as follows; Stage I 93%, Stage II 70%, Stage III 37% and Stage IV 25% (Trimble 1999).

Optimal debulking of ovarian cancer at the time of primary surgery is an important determinant of survival. Suboptimal debulking, leaving residual disease at the end of the surgical procedure is associated with adverse survival.

Of all the histological types of ovarian cancer, clear cell carcinomas have shorter survivals stage for stage than serous carcinomas but histological type per se is not a prognostic factor. In contrast, grade of tumour has been accepted as a prognostic factor (Vergote et al. 2001).

DNA ploidy has emerged as an independent prognostic factor in most but not all studies (But and Gorisek 2000; Kimmig et al. 2002; Nagai et al. 2001; Ozalp et al. 2001). Other pathological features such as measurements of cellular proliferative activity have not been found to be of prognostic value.

Measurement of serum levels of the cancer-associated antigen CA125 forms an integral part of the management of ovarian cancer. CA125 levels at the time of diagnosis are of limited prognostic significance but in patients with stage III and IV disease, an elevated level of CA125 after cytoreductive surgery and three cycles of chemotherapy has been shown to be an adverse and independent prognostic variable (van Dalen et al. 2000). Evaluation of molecular biological markers as prognostic factors in ovarian cancer has produced conflicting results. The most frequently detected molecular abnormality in ovarian cancers is mutation of p53 but its prognostic significance remains unclear. Overexpression of ErbB2 is observed in a proportion of ovarian cancers and evidence suggests that this correlates with adverse survival (Meden and Kuhn 1997).

Finally, performance status and age are strong predictors of disease outcome as shown by a meta analysis of trials involving over 2000 patients (Thigpen et al. 1993).

1.3.2 Staging and primary surgical treatment

In the absence of extra-abdominal metastatic disease, definitive staging of ovarian cancer requires laparotomy. Staging of ovarian cancer is carried out surgically and is important in determining prognosis and subsequent management. Laparotomy should be performed through a midline vertical incision. Surgical staging should include a thorough methodical assessment of the abdominal cavity. The exact extent of disease must be noted. Even if the disease appears to be limited to the ovaries or pelvis, it is essential to examine the diaphragm, liver, spleen, both paracolic gutters, the peritoneum, para-aortic and pelvic lymph nodes and omentum. If ascites is present a sample should be sent for cytological examination, otherwise peritoneal washings should be obtained. Table 1.1 shows the FIGO staging classification for ovarian cancer.

Surgery aims to reduce tumour cell volume as much as possible. The ideal outcome is that no macroscopic tumour is visible at the end of the procedure. Residual disease at the end of the procedure must be documented. If there is no residual macroscopic tumour at the end of the surgical procedure, the patient is said to be completely debulked. A procedure leaving residual tumour deposits < 2cm is classed as optimal debulking whilst a patient with residual disease >2cm would be described as suboptimally debulked. Prognosis in patients with ovarian cancer depends on the stage of tumour and efficacy of the debulking procedure. In a postmenopausal woman, it is standard procedure to carry out a hysterectomy, bilateral salpingo-oophorectomy and omentectomy. Management of

a premenopausal woman wishing to retain her fertility has to be highly individualised and based on intra operative findings.

Table 1.1 FIGO staging classification for ovarian cancer

FIGO STAGE	
I	**Growth limited to the ovaries**
Ia	Growth limited to one ovary, no ascites, no tumour on the external surface, capsule intact
Ib	Growth limited to both ovaries, no ascites, no tumour on the external surfaces, capsules intact
Ic	Tumour stage Ia or Ib but with tumour on the surface of one or both ovaries,with capsule ruptured,with ascites present containing malignant cells, or with positive peritoneal washings.
II	**Growth involving one or both ovaries with pelvic extension**
IIa	Extension or metastases to the uterus or tubes
IIb	Growth involving one or both ovaries with pelvic extension
IIc	Tumour either stage IIa or IIb but with tumour on the surface of one or both ovaries, with capsule ruptured, with ascites present containing malignant cells, or with positive peritoneal washings.
III	**Tumour involving one or both ovaries with peritoneal implants outside the pelvis or positive retroperitoneal or inguinal nodes, superficial liver metastases equal stage III, tumour limited to the true pelvis but with histologically verified malignant extension to small bowel or omentum**
IIIa	Tumour grossly limited to the true pelvis but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces
IIIb	Tumour of one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces, none exceeding 2cm in diameter, nodes negative
IIIc	Abdominal implants greater than 2cm in diameter, or positive retroperitoneal or inguinal nodes
IV	**Growth involving one or both ovaries with distant metastases**, if pleural effusion is present, there must be positive cytologic test results to allocate a case to stageIV, parenchymal liver metastases equals stageIV

1.3.3 Chemotherapy

It is generally accepted that surgery alone is adequate for stage1a and 1b if the tumour is well differentiated (Vergote et al. 2001). All other patients require some form of chemotherapy. Ovarian cancer in general is a chemosensitive disease. The gold standard first line chemotherapy is a combination of a platinum compound and a taxane. For patients with refractory or relapsed disease the choice of subsequent treatment must be tailored to the individual. In this group of patients, all treatment is palliative and therefore quality of life and toxicity of treatment are especially important considerations.

1.3.3.1 Platinum compounds

Cis-Diamminedichloroplatinum (II) or cisplatin is a highly effective antitumour agent used in the treatment of ovarian cancer. Cisplatin reacts with purines to form a variety of DNA adducts. The mechanism by which the cisplatin-DNA adducts induce cell death is not fully understood. The formation of both intrastrand and interstrand crosslinks induce structural distortions in DNA. These are thought to inhibit DNA replication and transcription and ultimately induce apoptosis. Cisplatin can cause severe nephrotoxicity and neurotoxicity. Carboplatin, an analogue of cisplatin produces less neurotoxicity but has myelosuppression as its dose limiting toxicity. Nevertheless, its equivalence of efficacy and lower toxicity have resulted in carboplatin largely replacing cisplatin in the clinic.

1.3.3.2 Taxanes

Paclitaxel was identified as the active anti tumour agent in the extract of bark from the pacific yew tree in 1971. A synthetic analogue, based on an extract of the European Yew

tree (Taxus baccata), docetaxel is now also available. The taxanes act by binding to microtubules that are integral components of the mitotic spindle. Binding of taxanes to the microtubules stabilizes them and prevents their depolymerisation. This inhibits cell proliferation by causing a metaphase arrest in dividing cells.

1.3.4 Drug Resistance

Drug resistance is a major obstacle limiting our ability to cure patients with ovarian cancer. Multiple mechanisms lead to drug resistance and resistance to any one drug is likely to be multifactorial. Resistance to chemotherapeutic drugs can be an intrinsic property of the tumour or can be acquired following treatment.

Reduced intracellular drug accumulation is an important mechanism by which cells become drug resistant. A family of transmembrane proteins has been identified that use an energy dependent process to transport drugs out of the cell. The first such protein identified was P-glycoprotein (Ueda et al. 1986). P-glycoprotein is the product of the MDR1 gene and is an ATP dependent, transmembrane transporter. This protein functions as a drug efflux pump. Cells that overexpress P-glycoprotein accumulate less drug resulting in insufficient intracellular drug levels to mediate cytotoxicity. Many tumours display a multidrug resistant phenotype and in many instances this has been shown to correlate with P-glycoprotein overexpression. Multi drug resistance associated protein, (MRP), is another of the family whose expression confers a similar but not identical drug resistant phenotype (Cole et al. 1994).

Enhanced inactivation of drug is another mechanism that can render a cell drug resistant. Elevated levels of intracellular glutathione and metallothioneins are associated

with drug resistance. Conjugation of these compounds with drugs produces a more water soluble less toxic product that is readily excretable.

Drugs such as platinum compounds and alkylating agents exert their cytotoxic effects by forming lesions in DNA. Following DNA damage, cells must repair or tolerate DNA damage to survive. The ability of tumour cells to repair DNA may be a mechanism of drug resistance. Enhanced DNA repair has been observed in cisplatin resistant ovarian cancer cell lines (Masuda et al. 1990). Other studies have identified a link between mismatch repair deficiency and cytotoxic drug resistance (Strathdee et al. 1999).

Alterations in expression of oncogenes (myc,ras,jun,fos,v-abl,Her/neu) can influence cellular sensitivity to cytotoxic drugs although the mechanisms of this resistance are uncertain. Since the expression of these oncogenes can lead to pleiotropic changes in cells, the mechanism of resistance may be multifactorial. Alteration of the tumour suppressor gene p53 may also influence chemosensitivity.

1.3.5 Natural history of ovarian cancer

The defining feature of a malignant as opposed to a benign tumour is the capacity for invasion. In the context of carcinomas, invasion involves breach of the basement membrane with infiltration of the underlying stroma. Following neoplastic transformation, the initial step in tumourigenesis is local multiplication of malignant cells. In order for a tumour cell to invade, it must first be able to detach itself from the primary tumour. This occurs by a reduction in tumour cell adhesiveness which is commonly associated with a reduction in expression of E- Cadherin (ECDH). ECDH is a calcium dependent cell to cell adhesion molecule whose expression has been shown to

be frequently downregulated in epithelial cancers (Risinger et al 1994). ECDH promotes cell to cell adhesion by connecting to the cytoskeleton via beta and alpha catenins. Degradation of the basement membrane and extracellular matrix by tumour cells is a prerequisite for invasion. Metalloproteinases are proteolytic enzymes produced by both connective tissue and tumour cells that can degrade the components of the extracellular matrix and basement membrane allowing tumour cell invasion. Following detachment and invasion, tumour cells must be able to reattach in their new location where they can then proliferate and or invade.

For tumours to survive, angiogenesis must take place. Growth factors secreted by both tumour and host cells stimulate angiogenesis.

Unlike many other cancers, epithelial ovarian cancers rarely form metastatic deposits outwith the abdominal cavity. Instead there is often extensive locoregional dissemination of disease within the abdominal cavity. Undoubtedly tumour cells reach the microvasculature of many organs but extravasation into the organ parenchyma and growth rarely occurs. The reasons for this phenomenon are unclear.

Altered expression of chemokine receptors in breast tumour cells has been postulated as a determinant of metastatic site (Muller et al. 2001). The lack of propensity of ovarian cancer to spread outside the abdominal cavity suggests some sort of mechanism whereby cells can survive only in the milieu of the peritoneum or perhaps a mechanism that inhibits growth outwith that body cavity. This may be due to due to lack of appropriate growth factors, pH, cell surface receptors or ligands.

1.4 HUMAN GENOME PROJECT

The human genome project (HGP) is a publicly financed international research effort whose goal is to decipher the human genetic code and provide this data freely and rapidly to the public. The HGP is a collaboration involving 20 groups from the United States, the United Kingdom, Japan, France, Germany and China. The intent of the collaborators was to make all the sequence data available without restriction within 24 hours of completion.

The idea of sequencing the human genome was first proposed at scientific meetings organized by the United States Department of Energy and others from 1984 – 1986. The United States National Research Council appointed a committee who endorsed the concept in its 1988 report. Rather than limiting its aim to obtaining just the sequence of the entire human genome, the committee recommended a broader programme which included the creation of genetic, physical and sequence maps of the human genome with parallel efforts in model organisms such as bacteria, yeast, worms, flies and mice. The committee also advocated research into the ethical, legal and social issues raised by human genome research.

By late 1990, the HGP had been launched and involved all the countries previously mentioned. The Human Genome Organization (HUGO) was also formed to provide a forum for international coordination of genomic research.

Initially, work focussed on construction of genetic and physical maps of the human and worm genomes. Sequencing of the yeast and worm genomes showed that large-scale sequencing was feasible and a two-phase paradigm for sequencing was developed. The first phase would produce a draft genome sequence and the second would be a finishing

phase in which gaps would be closed and remaining ambiguities resolved through directed analysis.

Initially pilot projects were launched to demonstrate that cost effective accurate large-scale sequencing was possible. These projects met their target completion date of March 1999 and from that time on the human genome sequencing project moved into full-scale production. The completion of a "working draft" of the human genome was announced in June 2000 and published in the February 15th 2001 issue of Nature (Lander et al. 2001).

To sequence the human genome, a hierarchical shotgun sequencing strategy was employed. A human genomic DNA library was constructed by fragmenting the genome with restriction endonucleases. The fragments (typically 100=200kb) making up the library were then organized into a physical map and non-redundant overlapping BAC clones selected for sequencing. The BAC clones were further endonuclease digested and the resulting fragments cloned and sequenced (random shotgun strategy). Finally, the clone sequences were assembled to reconstruct the sequence of the genome.

1.5 BIOINFORMATICS

With a rapid expansion in the volume and complexity of molecular biological and genetic data it was a requirement that computerised databases and analysis were developed to deal with this huge expansion.

In 1988, the National Center for Biotechnologies Information (NCBI) was created. It was formed as a division of the National Library of Medicine (NLM) at the National Institutes for Health (NIH). The stated mission of the NCBI was to develop new

information technologies to aid in the understanding of fundamental molecular and genetic processes that control health and disease. The NCBI was to be responsible for creating automated systems for storing and analysing knowledge about molecular biology, biochemistry and genetics. These systems were to be readily accessible to the research and medical community. In the early days of the human genome project the emphasis for the bioinformaticists was on the creation and maintenance of databases. Now with the release of the first draft of the sequence, the emphasis has shifted to analysis and interpretation of the data. A major part of this computational biology involves data mining. In this process, testable hypotheses are generated regarding the function or structure of a gene or protein of interest by identifying similar sequences in better characterised organisms.

The Cancer Genome Anatomy Project (CGAP) is an interdisciplinary program established and administered by the National Cancer Institute (NCI) to generate the information and technological tools needed to decipher the molecular anatomy of the cancer cell. The goal of this project is to determine the gene expression profiles of normal, precancer, and cancer cells, leading eventually to improved detection, diagnosis, and treatment for the patient.

From CGAP's inception in 1996, NCBI has been a key participant in bioinformatics planning, data tracking, data archiving, and analytical tool development and implementation. NCBI is collaborating closely with a component of CGAP called the Cancer Chromosome Aberration Project (CCAP). Using fluorescent in situ hybridization (FISH), CCAP is generating clones that are spaced 1-2Mb across the human genome. Once mapped, these sequence-ready DNA BAC clones are then made available to the

research community. NCBI is involved in identifying candidate BAC clones to be FISH-mapped, archiving the results, and localizing these clones onto draft sequence contigs. This data can be viewed through NCBI's Map Viewer and is linked to NCBI's Clone Registry and UniSTS sites.

CCAP is also characterizing chromosome aberrations in selected tumor types through the use of spectral karyotyping (SKY) and comparative genomic hybridization (CGH). SKY facilitates identification of chromosomal aberrations and CGH can be used to generate a map of DNA copy number changes in tumour genomes. The SKY/CGH database has been designed to house this data, and is publicly accessible. The Mitelman Database of Chromosome Aberrations in Cancer is a summary of all documented recurrent neoplasia-associated chromosomal aberrations. This work, which originally appeared in the April 1997 Special Issue of Nature Genetics is continuously updated and a summary of the latest data can be viewed with NCBI's Map Viewer.

Molecular biology researchers have an enormous range of bioinformatics programs and databases available to them over the internet. For the research undertaken in this thesis several programs have been particularly helpful. BLAST (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov:80/BLAST/) is a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA (Altschul et al. 1990). ExPASy Translate (http://www.expasy.ch/tools/dna.html) is a tool that allows the translation of a nucleotide (DNA/RNA) sequence to a protein sequence. A full discussion of all algorithms and databases is beyond the scope of this thesis but many of the programs used to facilitate

the work in this thesis were accessible through the NCBI home page (http://www.ncbi.nlm.nih.gov:80/).

1.6 MOLECULAR GENETICS OF OVARIAN CANCER

That cancer arises as a result of genetic alterations within cells has been known for a long time. Tumorigenesis is thought to be a multistep process involving the acquisition of multiple genetic alterations, which allows the cell to escape from the normal growth constraints/mechanisms in the cell. Alterations of both proto-oncogenes and tumour suppressor genes are involved in tumorigenesis.

Proto-oncogenes encode genes involved in cell signalling pathways associated with growth control. They can be converted into activated oncogenes by amplification, mutation or translocation.

Tumour suppressor genes act as regulators of cell growth and proliferation. They act recessively at the cellular level; that is to say that the function of both alleles must be disrupted before tumour suppressor activity is lost. Loss or inactivation of a tumour suppressor gene may be the result of several mechanisms including point mutations, deletions, chromosomal loss and epigenetic phenomena such as methylation.

1.6.1 Inherited predisposition to ovarian cancer

Whilst the majority of ovarian cancers are sporadic approximately 5-10% of cases are demonstrated to have an inherited predisposition as judged by autosomal dominant familial clustering. Three hereditary syndromes have been identified, namely hereditary site-specific ovarian cancer, hereditary breast and ovarian cancer and the type 2 Lynch

syndrome. Linkage analyses of the affected families have identified the susceptibility genes BRCA1 and BRCA2 on chromosome 17q and 13q respectively (Miki et al. 1994) (Wooster et al. 1995). Germline mutations of these genes can be found in the majority of cases of hereditary site-specific ovarian and hereditary breast and ovarian cancers. Both BRCA1 and BRCA2 have been demonstrated to be tumour suppressor genes.

Type 2 Lynch syndrome patients have mutations of certain mismatch repair genes and have a propensity to develop tumours from the colon, endometrium and ovary (Lynch et al. 1985b) (Lynch et al. 1985a).

1.6.1.1 BRCA1 and ovarian cancer

The BRCA1 gene was identified by positional cloning in 1994 (Miki et al. 1994). Located on chromosome 17q it encodes a large protein and spans 84kb of genomic sequence (Welcsh and King 2001). It contains 24 exons, the largest of which, exon 11 codes for more than 60% of the 1863 amino acid protein. The protein contains several functional domains including an N terminal RING finger domain and two BRCA1 terminal (BRCT) domains at the carboxy terminus.

Many lines of evidence confirm that BRCA1 is a tumour suppressor gene. Frequent loss of heterozygosity on chromosome 17q has been observed in familial and sporadic breast and ovarian cancers (Nagai et al. 1994) (Caduff et al. 1999). In accordance with Knudson's hypothesis, patients with germline mutations of the gene develop early onset disease, as they only require to sustain one hit in order to have inactivated both copies of the tumour suppressor gene. Early functional studies involving transfer of the long arm

of chromosome 17 into a breast cancer cell line demonstrated suppression of the tumorigenic phenotype (Negrini et al. 1994).

Much progress has been made in the functional analysis of BRCA1 and many of the proteins with which it interacts have been identified. BRCA1 plays an important role in DNA damage repair and transcriptional regulation.

The first evidence for BRCA1 involvement in DNA damage repair came from findings that BRCA1 interacts with RAD50 and RAD51 proteins that are implicated in DNA damage repair pathways (Chen, Lee, and Chew 1999). Subsequently, BRCA1 deficient embryonic stem cells have been shown to be hypersensitive to oxidative agents such as irradiation and display defects in double strand break repair (DSBR) (Deng and Scott 2000). Retrovirally expressed, wild type BRCA1 decreased the gamma radiation sensitivity and increased the efficiency of double strand DNA break repair of the BRCA1-/- human breast cancer cell line, HCC1937 (Scully et al. 1999). By contrast, multiple, clinically validated, mis-sense mutant BRCA1 products were non-functional in these assays and failed to rescue the phenotype. These data suggest that efficient repair of double strand DNA breaks is a key BRCA1 tumour suppressor function.

Dysfunction of BRCA1 in cells leading to abnormalities in DNA structure would be expected to provoke cell death at the point of cell checkpoint control. This can be seen to occur in BRCA1 homozygous mutant mouse fibroblasts on exposure to ionising radiation (Shen et al. 1998). If however, loss of function of BRCA1 occurred in a premalignant cell that had already acquired inactivation of key checkpoints, aberrant DNA structures resulting from BRCA1 inactivation might be tolerated without cell cycle arrest.

BRCA1 has also been shown to function as a transcription factor. Certain fragments of the protein when fused to a GAL4 DNA-binding domain can transactivate a GAL4 reporter except when they bear clinically relevant mutations (Chapman and Verma 1996). The C terminal region of BRCA1 contains the two BRCT domains that interact with multiple transcription activators and co-repressors. The second BRCT domain interacts with p53 and stimulates p53- dependent transcription of the p21 promoter (Chai et al. 1999). Regulation of p53 is another way in which BRCA1 can be seen to function in maintaining genomic stability.

The prevalence of BRCA1 mutations in western populations is between 0.03-0,01% (Easton, Ford, and Peto 1993). The Ashkenazi Jews have a much higher incidence of disease associated alleles in the order of 1% (Bahar et al. 2001).

More than sixty different germline mutations of BRCA1 have been identified (Xu and Solomon 1996). The mutations span the entire region of the gene and include deletions and insertions as well as mis sense, nonsense and splicing mutations. The vast majority of mutations such as the 185delAG in exon 2 in Ashkenazi Jewish populations result in the production of nonfunctional truncated forms of the protein product.

For carriers of a germline BRCA1 mutation, the estimated risk of ovarian cancer is 20-50% by age 70 years (Ford et al. 1998).The mean age of onset is about 5 years younger in hereditary than in sporadic ovarian carcinoma. Serous adenocarcinoma is the most common histological type (83% vs 49% matched controls) (Chang et al. 1995). Several studies have suggested that patients with ovarian cancer who are carriers of BRCA germline mutations have a better prognosis than those without mutations. A study by Y Ben David et al (Ben David et al. 2002) of patients with ovarian cancer found a

significant difference in survival between BRCA1/BRCA2 carriers and non-carriers. The reason why carriers had a better survival is not clear.

1.6.1.2 BRCA2 in ovarian cancer

BRCA2 was cloned in 1995 (Wooster et al. 1995). It is located on chromosome13q12-13 and spans 86kb of sequence. It encodes a protein of 3418 amino acids. BRCA2 has 27 exons and although it is unrelated in sequence to BRCA1 it also includes an unusually large central exon 11. Encoded within this exon are eight BRC repeats composed of approximately forty residues each. These repeats are the major sites for direct binding of BRCA2 to RAD51 which is involved in DNA repair (Wong et al. 1997).

BRCA2 appears to have very similar functions to BRCA1. It plays a role in DNA repair and has been implicated in the regulation of transcription. Frequent LOH on chromosome 13q12-13 in sporadic ovarian cancer has been documented (Gras et al. 2001).

More than one hundred disease causing mutations in BRCA2 have been identified. The majority of mutations are deletions and result in truncation of the BRCA2 protein.

In the largest study to date, the estimated cumulative risks of ovarian carcinoma in families carrying a mutation in BRCA2 were 0.4% by the age of 50 and 27% by the age of 70 (Ford et al. 1998). There is some data to suggest that an ovarian carcinoma mutational cluster region in exon 11 of BRCA2 confers higher risk of ovarian cancer (Gayther et al. 1997) but not all studies support this.

1.6.2 Oncogenes

Protooncogenes encode components of the signal transduction pathways involved in the regulation of cell growth. There are four main groups of protooncogenes encoding growth factors, growth factor receptors, signal transducers and nuclear transcription factors. Protooncogenes can be converted into oncogenes by insertion, amplification, mutation or translocation. Activation of oncogenes contributes to the uncontrolled cellular proliferation that is characteristic of malignancies.

Of the growth factor receptors, the epidermal growth factor (EGF)/ ErbB family has been extensively studied. The EGF/ErbB receptor family has four members: ErbB1 (HER-1, EGFR-1), ErbB2 (HER-2, NEU), ErbB3 (HER-3) and ErbB4 (HER-4). These receptors are comprised of an extracellular ligand binding domain, a hydrophobic transmembrane region and an intracellular portion encoding a tyrosine kinase domain. Ligand binding induces a conformational change in the receptor that stimulates receptor dimerisation that can be either homo or heterodimeric. Following dimerisation, activation of the tyrosine kinase domain of the receptor leads to phosphorylation and activation of regulators of downstream pathways.

Members of both the EGF family of peptides and the EGF family of receptors show altered expression in ovarian tumours. Overexpression of EGF-R1 in ovarian cancer has been well documented and antisense suppression of EGF-R expression has been shown to alter cellular proliferation, cell adhesion and tumorigenicity in ovarian cancer cells (Alper et al. 2000). Overexpression of HER-2 occurs in approximately 30% of ovarian cancers and is associated with poor survival (Berchuck et al. 1990).

Different EGF - like peptides are overexpressed in many human carcinomas compared with their untransformed counterparts. These tumour-derived growth factors can act as both paracrine and autocrine factors to stimulate cell proliferation and survival in cells that express the receptors for these peptides. Antisense oligonucleotides to EGF related peptides inhibit the proliferation of ovarian carcinoma cells (Casamassimi et al. 2000).

Discovery of an activated form of Ras in a human bladder cancer cell line was the first identification of a human oncogene. There are 3 human ras genes, Harvey, Kirsten and N-ras. The ras proteins bind guanine nucleotides and activate signalling cascades. They are located on the inner leaflet of the plasma membrane and in their basal state they are bound to GDP. Activated ras binds GTP and activates downstream signalling proteins until GTP hydrolysis mediated by the intrinsic activity of ras returns the system to its basal state. Ras mutations have been identified in many tumour types. Point mutations that activate ras have been found to cluster in the regions encoding codons 12 and 13 and 59-61. These mutations act by interfering with the GTP hydrolysis leading to constitutive activation of ras.

K-ras activation was found to occur frequently in mucinous adenocarcinomas but rarely in other common epithelial tumours of the human ovary (Enomoto et al. 1991). A later study, of benign, borderline and malignant mucinous tumours identified a frequency of codon 12/13 mutations of 68%. This study identified these mutations in all three groups of tumours (55,73, 85% respectively) suggesting that k ras mutational activation is an early event in mucinous ovarian tumorigenesis (Cuatrecasas et al. 1997) .A study of K-ras mutations in non mucinous ovarian epithelial tumours found an overall prevalence of mutations of 30.5%(Cuatrecasas et al. 1998). This is much lower than the prevalence of

mutations in mucinous tumours and suggests that K-ras mutations are not initial events in the pathogenesis of non-mucinous ovarian tumours.

The myc gene product is a nuclear protein. Myc is expressed at very low levels in quiescent cells but its expression is rapidly induced in response to mitogenic signals. The level of myc expression reflects the proliferation status of the cell. Regulation of expression of nuclear proto-oncogenes is intrinsic to their function and activation of nuclear oncogenes often occurs by deregulation of expression rather than mutation. Deregulated expression can occur as a result of translocation or amplification of the myc gene. Many studies have identified amplification and overexpression of c-myc in ovarian carcinomas (Diebold et al. 1996) (van Dam et al. 1994) (Berns et al. 1992) (Tashiro et al. 1992) (Baker et al. 1990) (Kohler et al. 1989). Rates of amplification and overexpression ranged from 17-48% amongst these studies. There was no apparent association with survival. One study identified a significant association of overexpression of c-myc in stage III ovarian cancers compared to other stages.

The serine/threonine protein kinase, protein kinase B or Akt (PKB/Akt) has been shown to play an important role in oncogenesis. In humans, there are three closely related Akt family members designated Akt1, Akt2, Akt3 located at chromosomes 14q32, 19q13 and 1q44 respectively. One of the first reports of Akt gene involvement in human cancer was demonstration of amplification and overexpression of Akt2 in ovarian tumours and cell lines (Cheng et al. 1992). The Akt kinases are downstream targets of growth factor receptor tyrosine kinases that signal via phosphatidylinositol 3-kinase (PI3K). PI3K generates phosphatidylinositol-345 triphosphate (PIP3) which is essential for the translocation of Akt to the plasma membrane where it is phosphorylated and activated

by phosphoinositide-dependent kinase-1 (PIDK1). The tumour suppressor PTEN inhibits Akt activation by dephosphorylating PIP3 (Tamura et al. 1999). Constitutive activation of Akt in cancers has been shown to result from amplification of Akt or as a result of mutations in components of the signalling pathway that activates Akt. Akt appears to both negatively regulate factors that promote the expression of death genes and positively regulate factors that induce survival genes (Nicholson and Anderson 2002).

1.6.3 Cytogenetics

Whilst linkage analysis has been used to identify genes involved in cases of inherited cancer, different approaches are used to investigate the somatic genetic alterations occurring in sporadic cancers which account for approximately 90-95% of ovarian cancer cases. Cytogenetic studies can identify frequent non-random structural genetic alterations of chromosomes within tumour cells suggesting specific chromosome regions on which to focus to identify genes involved in the cancer process.

Many studies performing cytogenetic analyses of ovarian cancers have been published (Brock et al. 1996; Kiechle-Schwarz et al. 1995; Nacheva et al. 1998; Pejovic 1995; Rao et al. 2002). These studies have indicated the presence of multiple complex chromosome abnormalities in ovarian cancer. The largest study to date is a cytogenetic analysis of 244 primary ovarian cancer specimens (Taetle et al. 1999). This study identifies non-random patterns of chromosome breakpoints at the level of chromosome regions. Simultaneous occurrence of multiple abnormalities was common but nearly every tumour with chromosome breaks had breakpoints in one of thirteen non-randomly involved regions from chromosomes 1, 6, 7, 11, 12, 13 and 19.

Comparative genomic hybridisation and fluorescence in-situ hybridisation are alternative methods used to identify structural genetic abnormalities.

1.6.4 Loss of heterozygosity in ovarian cancer

The finding of a region of frequent loss of heterozygosity (LOH) in tumour DNA suggests the possibility that a tumour suppressor gene may reside at that locus. To date, many studies of LOH in epithelial ovarian cancer have been performed and several hotspots of LOH have been identified. There are however, many obstacles to be overcome in the interpretation of LOH data. Despite the large number of studies of LOH it is difficult to perform a meaningful meta-analysis of the results. Studies vary as to their inclusion of benign, borderline and malignant tumours. Variation between studies with respect to clinicopathological variables such as histology and stage further complicates attempts at data comparison. Many studies contain only a small number of tumours and study findings often fail to reach statistical significance. Another factor that affects LOH findings and hinders comparison of data is the choice and number of microsatellite markers examined. Some studies report presence or absence of LOH based on analysis of only a small number of markers, sometimes only one or two markers per chromosome arm. Furthermore, rates of LOH may be influenced by the use of microdissected or undissected tumour tissue. Studies using undissected tissue may fail to detect LOH that is masked by a high percentage of normal stromal tissue in the sample. Finally, definition of what constitutes LOH is an arbitrary one. Commonly LOH is assigned where the difference in the ratio of normal to tumour alleles varies by greater

than thirty percent ($r < 0.7$). Some authors however have used a more stringent cut off, only assigning LOH where $r < 0.5$.

To date greater than 100 studies of LOH in ovarian cancer have been published. LOH on almost every chromosome has been identified. The most commonly reported LOH findings in ovarian cancer are LOH at 17p and 17q, the sites of the p53 and BRCA1 tumour suppressor genes respectively.

1.6.5 p53 - THE PARADIGM OF A TUMOUR SUPPRESSOR GENE

1.6.5.1 p53 The paradigm of a tumour suppressor gene

The p53 tumour suppressor protein is a multifunctional protein. It is a transcription factor whose 2 main functions are the induction of cell cycle arrest and apoptosis.

The p53 protein has been labelled the "guardian of the genome". p53 responds to signals produced by a wide range of cellular stresses such as heat, hypoxia, hyperoxia, cytokines, growth factors, metabolic changes, cell to cell contact and activated oncogenes. P53 responds to these insults by activating a set of genes whose products facilitate adaptive and protective activities that include cell cycle arrest and apoptosis.

Cellular stresses such as DNA damage cause p53 accumulation. The factors that determine whether cell cycle arrest or apoptosis ensues after p53 activation are not understood. The outcome is likely to be cell type specific and contextual. Cell cycle arrest at the G1 and G2 checkpoints prior to DNA replication and mitosis allows time for DNA repair thus preventing the propagation of mutations and aneuploidy. On the other hand, apoptosis is a fail-safe mechanism to rid the organism of cells with severely damaged DNA. Transcriptional activation of the p21 WAF/CIP1 cyclin-dependent

kinase inhibitor plays a key role in the induction of cell cycle arrest but there does not seem to be a similar single critical apoptotic target.

The p53 gene is located on the short arm of chromosome 17 in a region, which is subject to loss of heterozygosity in many different cancers. The p53 gene spans a genomic distance of more than 20kb and has 11 exons. The 393 amino acid protein has several functional domains and five regions that are highly evolutionarily conserved. (>90% homology between man and rodents in those conserved regions). Starting at the N terminal of the protein, the functional domains include a transcriptional activation domain, an SH3 binding domain, a sequence specific DNA binding domain, a tetramerization domain and a C-terminal basic domain.

In the context of specific binding to a DNA sequence, a region at the acidic N terminal of the protein forms a transcriptional activation domain which allows the p53 protein to recruit the basal transcriptional machinery required for mRNA synthesis. This region forms interactions with other proteins such as mdm2 and is therefore critically involved in regulating the stability and activity of p53. Interaction with mdm2 targets p53 for ubiquitin mediated degradation. Mdm2 binding also blocks the ability of p53 to interact with the transcriptional apparatus. The core of the p53 protein is the sequence specific DNA binding domain. The binding to DNA is optimal when the protein is in a tetrameric state which results from an interaction of four separate p53 molecules via the tetramerization domain adjacent to the C terminal region. The C terminal region is composed of predominantly basic residues and is involved in nuclear localisation and DNA damage recognition. The DNA binding domain is separated from the transcriptional activation domain by the SH3 binding domain. The SH3 binding domain

contains a series of repeated proline residues typical of a polypeptide that interacts with signal transduction molecules that contain an SH3 binding domain.

1.6.5.2 P53 in cancer

P53 participates in a cell cycle checkpoint signal transduction pathway that causes either a G1 arrest or apoptotic death after DNA damage or alternatively non genotoxic stress. Loss of p53 function would result in inappropriate progression through the cell cycle after DNA damage allowing survival of a cell that would otherwise have been destined to die. It is apparent therefore how loss of normal p53 function contributes to malignant transformation by causing increased genetic instability and a reduction in apoptosis.

P53 is the most commonly mutated gene in human cancers with at least 50% of all tumours having abnormal p53 genes. Some tumours develop other mechanisms of inactivating p53 such as overexpression of the p53 binding protein mdm2 or by infection with human papilloma virus (HPV). Expression of the HPV E6 protein binds p53 and enhances its degradation.

As well as being frequently mutated in sporadic cancer, germline mutations in p53 result in Li- Fraumeni syndrome, a hereditary cancer susceptibility syndrome predisposing individuals to sarcomas, lymphomas, breast, brain and other tumours. In accordance with these findings, mice deficient for p53 are highly tumour susceptible (Donehower et al. 1996).

In a study of p53 mutations in a range of human cancers, 98% of 280 base substitution mutations identified were found to occur in a 600bp region spanning exons 5-9. This

sequence contains a large number of evolutionarily conserved amino acids (Hollstein et al. 1991).

The location and type of mutations in a specific sequence define a mutational spectrum. By defining a p53 mutational spectrum for all cancer types, mutational hot spots can be identified. When mutations are examined for individual cancer types, clear differences in mutational spectra emerge. These spectra vary with respect to the position of the hotspots and with respect to the frequency of transitions (in which a purine is substituted for a purine or a pyrimidine for a pyrimidine) and transversions (in which a purine is substituted for a pyrimidine or vice versa.)

Different carcinogenic agents have been shown to produce different types of mutation and therefore analyses of mutational spectra allow us to generate hypotheses concerning the aetiology and molecular pathogenesis of cancer. G:C to T:A transversions in p53 have been shown to result from polycyclic aromatic hydrocarbon exposure and are commonly seen in lung cancers. G:C to A:T transitions are the most common p53 mutations seen in colon cancer. This mutation is the result of endogenous mutational mechanisms caused by deamination of 5methyl cytosine residues found at CpG dinucleotides. The most striking p53 mutational spectrum is found in hepatocellular carcinoma arising in people living in geographic areas where aflatoxinB and hepB virus are major risk factors for liver cancer. In these patients the majority of hepatocellular carcinomas carry a G:C to T:A transversion at the 3rd base position of codon 249. This transversion is not found in hepatocellular cancer arising in people living outwith regions of aflatoxinB exposure. This finding implicates aflatoxinB as the aetiological factor for this mutation. Another example of the mutational specificity of exposures

includes skin carcinoma and UV light yielding distinctive CC to TT transitions (Brash et al. 1991).

1.6.5.3 P53 and ovarian cancer

Alterations of the p53 gene appear to play an important role in the development of human ovarian cancers. Frequent LOH on 17p in ovarian cancer has been demonstrated and frequent mutations of p53 in ovarian cancer have been documented (Okamoto et al. 1991). As in other cancer types, the majority of mutations are found between exons 5-9. There is no consensus between the many studies regarding the nature of the mutations in p53 in ovarian cancer. Detection of varying proportions of transitions, transversions, insertions and deletions from one series to another may be due to environmental or endogenous influences or it may be due to the methods employed for detection of p53 mutations. Some studies have analysed only exons 5-9 for mutations whilst others rely on immunohistochemistry to screen tumours prior to mutation analysis. Normally, p53 is not immunodetectable. Mis-sense mutations of the p53 gene result in proteins that have a longer half-life than the wild type protein and are resistant to degradation. Some investigators have therefore used identification of p53 expression by immunohistochemistry as an indicator of the presence of mutated p53. This however means that tumours with p53 mutations that create an unstable protein product will not be detected.

The prognostic significance and nature of p53 dysfunction in ovarian carcinoma is uncertain. The relation between p53 expression, p53 mutations and overall survival has been investigated in many studies but small study size and technical difficulties have

made it difficult to draw conclusions about the prognostic implications of p53 mutations. A study by Fallows found that p53 mutation does not affect prognosis in ovarian epithelial malignancies (Fallows et al. 2001). In the largest study of its kind to date, of 171 epithelial ovarian cancers, 48.5% and 57.3% of the samples showed p53 overexpression and p53 mutation respectively (Shahin et al. 2000). Although neither p53 overexpression nor the mere presence of a p53 mutation impacted on overall survival, the combination did reveal adverse prognosis in both univariate and multivariate models. Studies have been performed to investigate the use of identifying p53 mutations as a predictor of chemoresistance. P53 has been shown to have a critical role in the induction of apoptosis following DNA damage caused by cytotoxic drugs. Loss of function of p53 may therefore be a determinant of chemoresistance in tumours. Sato et al showed that p53 dependent apoptosis in tumours was strongly related to chemosensitivity in epithelial ovarian cancer (Sato et al. 1999).

1.6.6 NOEY2

NOEY2 (ARH1) is a member of the Ras superfamily of small G proteins that was mapped to chromosome 1p31 (Yu et al. 1999). NOEY2 is expressed in normal breast and ovarian epithelial cells but not in breast or ovarian cancers. NOEY2 is expressed monoallelically and is imprinted maternally. In one study, LOH of the gene was detected in 41% of breast and ovarian cancers and in most of the cancer samples with LOH, the non-imprinted functional allele was deleted (Yu et al. 1999). Re-expression of NOEY2 through transfection suppresses clonogenic growth of breast and ovarian cancer cells. Overexpression of NOEY2 inhibits lactation and growth in transgenic mice (Xu et al.

2000). NOEY2 thus appears to be a putative imprinted tumour suppressor gene whose function is abrogated in breast and ovarian cancers. Unpublished data suggests that expression of NOEY2 may truncate signalling through ras/mitogen – activated protein kinase induced by growth factors (Yu et al. 1999). In a small study, genetic analysis of NOEY2 by SSCP in eighteen cancer cell lines and four normal controls revealed one germline mutation in the promoter region but no coding sequence mutations.

1.6.7 LOH on 11q in ovarian cancer

LOH on the proximal portion of 11q has been described but at lower frequencies than LOH on distal 11q and therefore will not be considered further. LOH of the 11q23-qter in epithelial ovarian cancer was first identified by Foulkes et al in 1993 (Foulkes et al. 1993). LOH was more common in advanced grade tumours but due to small study size this finding did not reach statistical significance. A study by Gabra et al identified allele imbalance at D11S912 to be associated with adverse survival and noted a trend towards significance for the association of LOH at D11S912 and Figo stage III/V disease (Gabra et al. 1995). A further study by Davis et al in 1996 identified two regions of LOH, the first region proximal to 11q24 and a second region lying between D11S912 and D11S439 (Davis et al. 1996).

The authors suggested that LOH of this second region was an early event in ovarian tumourigenesis because LOH was observed frequently in grade one and stage one tumours (as well as being a common event in higher grade and stage tumours). Concurrently, an independent study of LOH on chromosome 11q in epithelial ovarian cancer by Gabra et al refined the authors' previously defined region of LOH to an

overlapping 8.5 Mb region on 11q24 (Gabra et al. 1996). Loss of this region was associated with adverse survival. In 1998, Winqvist et al found that LOH at D11S912 was correlated with a higher tumour stage, serous histology and finding of residual tumour but not directly with survival times (Launonen et al. 1998). The lack of association with survival is contrary to findings of Gabra et al (Gabra et al. 1995) (Gabra et al. 1996).

1.6.8 Candidate tumour suppressor genes from chromosome 11q

Genes from 11q that have been investigated as candidate tumour suppressor genes include the ATM gene and PPP2RIB genes. Both these genes reside in a region of frequent loss of heterozygosity but neither have been found to be somatically altered in ovarian cancer suggesting that they are not the tumour suppressor genes from this region (Koike et al. 1999) (Campbell and Manolitsas 1999) (Wu et al. 1999). Located in the 11q24-5 region is Barx2, a homeodomain containing transcription factor that regulates the transcription of specific cell adhesion molecules in the mouse. Barx2 is expressed in the normal ovarian surface epithelium and has been shown to have functional tumour suppressor properties in ovarian cancer cells (Sellar et al. 2001). Using a combination of DHPLC and fluorescent SSCP, no mutations were detected in DNA from a panel of 71 cell lines (16 ovarian and 55 non-ovarian cell lines) and 12 ovarian tumours. (Unpublished data). Analysis of the methylation status of the Barx2 CpG island revealed that Barx2 is not methylated in cancer cell lines or ovarian tumours. (Unpublished data). These data suggest that Barx2 is not the 11q24-5 tumour suppressor gene.

1.7 METHYLATION AND CANCER

Methylation of the CpG islands associated with genes has been identified as a mechanism of inactivation of tumour suppressor genes in cancer. Methylation is an epigenetic process that produces a heritable change in DNA that does not alter the nucleotide sequence. Methylation of CpG islands is associated with the transcriptional silencing of the affected genes.

CpG islands are found at the 5' ends of most genes. They are short stretches of DNA with a high CG content and a high frequency of CpG dinucleotides relative to the rest of the genome. 95% of CpG islands are less than 1800bp long. (Lander et al. 2001) The most recent prediction of the number of CpG islands in the genome is 28890 (Lander et al. 2001) .CpG islands often include the promoter region of the associated gene as well as part or all of the first exon of the gene (Cross and Bird 1995). In contrast to the DNA outside of these CpG islands, the CpG dinucleotide in the island is unmethylated. Under normal circumstances there are only two exceptions to this lack of methylation. Imprinted genes, and genes on the inactivated X chromosome in females are methylated at their CpG islands. This suggests that lack of methylation is a prerequisite for transcriptional activation.

The mechanisms by which promoter hypermethylation repress transcription are not clear. The presence of a methylated cytosine may directly inhibit transcription factor binding but this would only affect factors with a CpG in their binding site. Methylation has been shown to alter chromatin structure and thereby affect access of transcription factors to their binding sites. Several proteins have been identified that bind preferentially to methylated cytosines in DNA. Methyl CpG-binding protein 2 (MECP2)

was the first member of this family of methyl CpG binding domain (MBD) proteins to be identified (Lewis et al. 1992). These proteins form complexes with proteins that include histone deacetylases and other transcriptional repressors. Recruitment of a histone deacetylase to the DNA leads to chromatin remodelling which alters transcriptional activation potential.

In transcriptionally active chromatin, acetylation of the positively charged lysine residues in the histones of the nucleosome promotes a relaxed open chromatin structure. This permits transcription by allowing transcription factors and RNA polymerase access to regulatory regions of the DNA. Deacetylation causes the chromatin to adopt a more closed compact formation that excludes access to transcription factors. The identification of MBD proteins provided a mechanism whereby methylation could influence transcription by recruiting proteins that alter acetylation status. Acetylation status undoubtedly influences the transcriptional status of genes but a study by Cameron et al showed that treatment with an inhibitor of deacetylase alone was not sufficient to reactivate expression of a gene silenced by methylation (Cameron et al. 1999). Only with the combination of TSA and azacytidine was the gene able to be reexpressed suggesting that in this context, methylation was the dominant silencing mechanism.

The importance of CpG island hypermethylation can be appreciated by considering the frequency of this process in cancer, the nature of the genes involved and the similar selective advantage conveyed by methylation as by mutations in coding regions. Several tumour suppressor genes that cause familial cancers through germline mutations are inactivated in association with promoter hypermethylation in sporadic cancers. These include VHL (Herman et al. 1994) p16 (Merlo et al. 1995), E- cadherin (Yoshiura et al.

1995) hMLH1 (Herman et al. 1998) and BRCA1 (Esteller et al. 2000). Until it was discovered that BRCA1 was frequently methylated in sporadic breast and ovarian cancers it was intriguing that mutations in the BRCA1 gene in the sporadic forms of these diseases were rare. The existence of promoter methylation of BRCA1 provided an alternative method of inactivation of this gene in the non-familial cases. A study by Esteller at al of DNA methylation in hereditary human cancers found similar patterns to those in sporadic tumorigenesis (Esteller et al. 2001). Analysis of the methylation of the CpG islands of 10 genes in inherited and non inherited breast and colorectal cancers showed that singly retained alleles of germline mutated genes were never hypermethylated in inherited tumours. However, in tumours where both alleles were retained, methylation of the wild type copy was a frequent second hit.

The mechanisms that lead to aberrant methylation of CpG islands have yet to be elucidated. In normal tissues, the DNA methyl transferases are a family of enzymes that catalyse the conversion of cytosine to 5' methyl cytosine. DNAmethyltransferase 1 (DNMT1) is the enzyme responsible for the maintenance of methylation and is constitutively active in proliferating cells. DNMT3A and DNMT3B are de novo methyltransferases that catalyse the transfer of a methyl group to previously unmethylated DNA. All 3 enzymes have been found to be overexpressed in tumour versus normal tissues with DNMT3B showing the highest overexpression (Robertson et al. 1999).

1.8 CELL ADHESION MOLECULES AND THE EXTRACELLULAR MATRIX

Cell adhesion is a fundamental property of multicellular organisms. Adhesion however does more than merely hold cells together. Cell to cell and cell to ECM adhesion have been shown in some systems to be required to block programmed cell death. This suggests that the physical processes of cell adhesion are linked to cellular signal transduction processes that control cellular differentiation, division and death. Adhesion therefore can determine the phenotype and fate of the cell. There are four main groups of cell adhesion molecules. Cadherins are the major cell to cell adhesion molecules with integrins, selectins and immunoglobulin cell adhesion molecules forming cell to cell and cell to ECM interactions. The majority of cell adhesion molecules are transmembrane glycoproteins. The extracellular component of the molecule interacts with cell adhesion molecules on neighbouring cells or with proteins of the ECM. At the intracellular surface of the cell membrane, cell adhesion receptors associate with cytoplasmic plaque or peripheral membrane proteins. These proteins link the adhesion system to the cytoskeleton and transduce signals initiated at the cell surface by the adhesion receptors. Many cells express a variety of cell adhesion molecules and individual cell adhesion molecules will not act in isolation. It is likely that a combination of many cell adhesion interactions determines the phenotype of a cell.

1.8.1 Cadherins

1.8.1.1 Cadherins in normal tissues

The cadherins are adhesion molecules that mediate homophilic calcium-dependent cell to cell adhesion. There are at least six subfamilies of the cadherin superfamily. In

general, cadherins comprise a large extracellular domain, a single transmembrane region and a cytoplasmic tail. E-Cadherin is studied as the prototype molecule for the cadherin superfamily. The extracellular portion of this molecule is made up of 5 subdomains, commonly designated EC1-5. Each subdomain comprises approximately ten amino acid residues (Takeichi 1995). Cadherin cell adhesion is dependent on calcium ions that bind to the extracellular portion of the molecule stabilising the cadherin structure. Cadherin mediated intercellular adhesion occurs in a zipper like fashion by interaction of the cadherin subdomains on one cell with cadherin subdomains on a neighbouring cell. For cadherins to be fully functional as cell adhesion molecules they require structural linkage to the cytoskeleton of the cell. This is achieved by binding of the cytoplasmic domain of the cadherin to catenin proteins. The catenin proteins bind to actin filaments thus acting as intermediate linker proteins.

Cadherins have been shown to be key regulatory molecules during development. The expression patterns of cadherins correlate with distinct morphogenetic events such as blastomere compaction, epithelial-mesenchymal conversion and folding of epithelia. Both over-expression and loss of function studies have been shown to lead to perturbations in morphogenesis. E-cadherin deficient mouse embryos cannot develop normally into blastocysts (Riethmacher, Brinkmann, and Birchmeier 1995) (Larue et al. 1994)) and mice without functional N-cadherin have abnormal heart and neural tube development. Studies in developing embryos have shown that expression of cadherins is dynamic. Altering the subclass of cadherin expressed or terminating the expression of an individual cadherin in a tissue is associated with its separation from the parent tissue e.g. Formation of the neural tube or formation of the mesoderm.

Through their interactions with the catenins, cadherins are postulated to influence intracellular signalling (Behrens 1999). It is impossible however to separate their role as mediators of cell adhesion from their function as mediators of cell signalling. As well as providing a link between cadherins and the cytoskeleton, beta catenin is part of the WNT signalling pathway. Beta catenin is normally found in complex with cadherins or as non-sequestered free beta catenin. This free cytoplasmic pool is usually rapidly phosphorylated and subsequently degraded. Accumulation of free cytoplasmic beta catenin results in its translocation to the nucleus. In the nucleus beta catenin binds to members of the T cell family of transcription factors and activates transcription of target genes that have oncogenic effects. Binding of beta catenin at the cell membrane by cadherins will reduce the pool of free cytoplasmic beta catenin thus decreasing its translocation to the nucleus with a consequent decrease in beta catenin signalling.

Cadherins exist alone in the cell membrane or as part of a junctional accumulation of cadherins such as in the zonula adherens of epithelial tissues. Biochemical and immunohistochemical studies have shown that the zonula adherens contains many proteins in addition to the cadherin-catenin complexes. The presence of other proteins suggests a mechanism whereby cadherin based adhesion may influence cellular signalling other than via the catenins. Signalling molecules such as members of the src family of non receptor tyrosine kinases are concentrated in adherens junctions and are good candidates to mediate adhesion dependent signalling events, although as yet, there is little evidence for this.

1.8.1.2 Cadherins and cancer

The majority of human cancers arise from epithelial tissues. E-cadherin is expressed in almost all epithelial tissues, where it is the most abundant adhesion molecule in adherens junctions (Gumbiner 1996). Expression of E-cadherin has been demonstrated to be reduced or absent in many different types of carcinomas including those of the female genital tract (Inoue et al. 1992). It has long been known that cell to cell adhesion is dramatically altered during the development of malignancy. Loss of E-cadherin cell to cell adhesion is concomitant with progression towards malignancy but it was a matter of debate as to whether loss of E-cadherin is a cause or effect of tumour progression. Its role as a tumour suppressor gene has now been firmly established. The role of E-cadherin as an invasion suppressor has been well documented in vitro (Behrens 1999) and direct evidence for a role of E-cadherin in the progression from benign adenoma to malignant carcinoma has been observed (Perl et al. 1998). It has been shown to be frequently mutated in cancers of the stomach and breast but is only rarely mutated in carcinomas of the ovary or endometrium (Risinger et al. 1994) (Berx et al. 1998). The location of E cadherin on chromosome 16q22 is subject to LOH in many cancers. Methylation of the CpG island of E Cadherin has been demonstrated to be a common method of allelic inactivation in many tumours including breast (Cheng et al. 2001) gastric (Machado et al. 2001), eosophageal (Si et al. 2001). prostate (Kallakury et al. 2001), renal cell (Nojima et al. 2001), oral squamous cell (Nakayama et al. 2001), hepatocellular (Matsumura, Makino, and Mitamura 2001) and thyroid carcinomas (Graff et al. 1998). Transient loss of expression of E-cadherin secondary to epigenetic silencing by methylation rather than disruption of a functional protein resulting from a

coding region mutation may be advantageous to the malignant cell. Transient loss of expression of E-cadherin may allow invasion but subsequent demethylation and reexpression may facilitate cell survival within metastatic deposits. Graff et al demonstrated that methylation patterns of the E-cadherin CpG island in breast cancer cells were unstable and reflected the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression (Graff et al. 2000).

1.8.1.3 Cadherins and ovarian cancer

Despite its wide acceptance as a tumour suppressor gene, it has been proposed that E-cadherin expression in the ovarian surface epithelium is necessary during the early stages of its neoplastic transformation. Normal human ovarian surface epithelium has very little if any expression of E-cadherin (Sundfeldt et al. 1997) In contrast, benign, borderline and malignant epithelial ovarian tumours have substantial expression of E-cadherin. Although arising from a simple mesothelium, epithelial ovarian tumours exhibit varying degrees of Mullerian differentiation and exhibit properties of oviductal, endometrial and cervical epithelia. It is believed that this more complex differentiated phenotype is mediated by the expression of E-cadherin (Auersperg et al. 1999). The requirement for E-cadherin expression in the development of ovarian neoplasia was investigated by transfecting immortalised normal human surface ovarian epithelial cells with E-cadherin (Ong et al. 2000). This resulted in the production of an ovarian adenocarcinoma cell line suggesting that E-cadherin was instrumental in promoting the transition to the malignant phenotype. Little is known about the role of other cadherin family members in the normal ovarian surface epithelium and in ovarian tumours. The

H-cadherin gene (CDH13) is located in a region of frequent deletion on chromosome 16q in ovarian cancer (Kawakami et al. 1999). The authors of this study detected a low expression of H-cadherin in the tumours with LOH of a polymorphic satellite marker within the H-cadherin gene. Methylation specific PCR identified methylation of H-cadherin alleles and they suggested that a combination of hyper-methylation and deletion cause the inactivation of H-cadherin in ovarian tumours. An immunohistochemical study of the expression of E and N-cadherins in ovarian tumours, detected N-cadherin expression in most benign and borderline tumours but expression was absent or heterogeneous in most carcinomas (Darai et al. 1997). E-cadherin was homogeneously expressed in benign tumours but was absent or heterogeneously expressed in most borderline and malignant tumours. A separate immunohistochemical study found that serous and endometrioid tumours express both E and N-cadherin. In contrast, mucinous tumours strongly expressed E-cadherin but not N-cadherin (Peralta Soler et al. 1997). K-cadherin (CDH6) and a potential regulator of its expression, Barx2, are expressed in the normal ovarian surface epithelium (Sellar et al. 2001). Sellar et al report significantly lower expression of both Barx2 and K-cadherin in a clinical sample of endometrioid and clear cell ovarian tumours as compared with serous or mixed mesodermal tumours. Barx2 has been postulated to be a tumour suppressor gene and it may exert its tumour suppressor functions in part, by modulation of K-cadherin expression.

1.8.2 Integrins

1.8.2.1 Integrins in normal tissues

Unlike other cell adhesion molecules the integrin family of cell adhesion molecules are heterodimers. Each heterodimer is composed of one alpha and one beta subunit. Both alpha and beta subunits are transmembrane proteins. So far at least 18 genes for alpha subunits and 8 genes for beta subunits have been identified (Hynes 1999). Theoretically a large number of alpha/beta combinations are possible but only just greater than 20 different combinations have been described. Integrins are transmembrane proteins that have a large extracellular domain, a membrane spanning region and a proportionally smaller cytoplasmic domain. Most integrins are predominantly receptors for ECM proteins (collagen, laminin and fibronectin) but a few have a role in cell to cell adhesion. This type of interaction is seen on leucocytes where they bind to receptors of the immunoglobulin superfamily (Walsh et al. 1996) or in one case, a cadherin, (Higgins et al. 1998). Integrins are expressed on a wide variety of cells and most cells express several integrins. The specificity of ligand binding by individual integrins is produced by the association of the extracellular, N terminal domains of the alpha and beta subunits to form a globular ligand-binding head on each integrin. This globular head binds the extracellular matrix ligand or the counter receptor. For integrins, the specificity in cell adhesion is only partially understood. Some integrins recognise the peptide sequence; arg-gly-asp (RGD) but for others this is not the preferred sequence. It is unclear how different RGD specific integrins discriminate between different RGD containing proteins. It is likely that the RGD site represents only part of or one of the integrin binding sites.

Integrins are the main cell adhesion molecules for the extracellular matrix proteins. They provide a link between the extracellular matrix and the actin containing filaments

of the cytoskeleton. They interact with talin and alpha actinin which in turn link with vinculin, tensin and other proteins linking to the actin cytoskeleton. Over the last few years however, it has become clear that integrins have many more roles than simply that of maintaining tissue architecture. Through their binding of the extracellular matrix, integrins play an important role in regulating many fundamental cellular processes. These include cell survival and proliferation, cellular differentiation and cell migration.

Integrins have been demonstrated to play important roles in signal transduction and cell signalling. They participate in bi-directional signalling referred to as inside-out and outside-in signalling, (Hynes 1992). Integrins also modulate signals initiated through other receptor types, particularly receptor tyrosine kinases that are activated by polypeptide growth factors. Sundberg et al described an integrin-mediated activation of PDGF beta that is independent of ligand (Sundberg and Rubin 1996). Integrin signalling and signal modulation involves binding of the integrin to its ligand, lateral clustering of integrins in the plane of the membrane and the formation of integrin-cytoskeletal complexes. This brings many different molecules into close proximity.

By selectively expressing different integrins, individual cells can vary their adhesive properties. Further variation in cell adhesiveness is achieved by the ability of cells to modulate the binding properties of integrins. This modulation of binding affinity of integrins arises in response to cytoplasmic signals and is known as inside out signalling. Intracellular signals are proposed to induce conformational changes in the cytoplasmic domain of the integrin. These changes are propagated to the extracellular ligand binding head of the integrin and binding affinity is thereby altered (O 'Toole 1994).

It has long been known that most types of normal cells require attachment to a substrate to be able to grow and that this dependence on anchorage is reduced in malignantly transformed cells (Folkman and Moscona 1978). Anoikis (Frisch and Francis 1994) is the programmed cell death caused by loss of anchorage of certain cell types to the extracellular matrix. As the main receptors for ECM proteins the integrins were implicated in the inhibition of anoikis and have subsequently been shown to be involved. Apoptosis of a differentiated epithelial cell that has been detached from its underlying matrix prevents the cell from establishing itself in a new and inappropriate location. This acts as an inbuilt safeguard to the host to prevent aberrant ectopic cell growth.

In neoplastic cells, alterations in integrins could lead to anoikis resistance thus facilitating anchorage independent growth or growth at an inappropriate site. Loss of cell-ECM interactions results in the induction of apoptosis in epithelial and endothelial cells (Meredith, Fazeli, and Schwartz 1993) (Frisch and Francis 1994). Loss of adhesion between fibroblast and ECM results in arrest of the fibroblasts in the G1 phase of the cell cycle (Guadagno et al. 1993). Many studies have shown that integrin engagement is necessary for cell cycle progression. Elevation of cyclin kinases has been shown to occur in response to integrin binding with concomitant reduction of cell cycle inhibitors (Fang et al. 1996) (Zhu et al. 1996). Thus it can be seen that outside-in signalling of integrins is a determinant of cell growth and survival.

Integrin binding has been shown to contribute to many intracellular signalling events that are implicated in the regulation of cellular growth, differentiation and survival. Integrin initiated signals stimulate the tyrosine phosphorylation of a number of cellular proteins. The best characterised of these is the phosphorylation and activation of focal

adhesion kinase (FAK). Activated FAK interacts with several proteins that lead to intracellular signalling. Activated FAK also binds to the structural proteins paxillin and tensin, which regulate the organisation of the actin cytoskeleton. Integrin binding leads to the activation of the mitogen activated protein kinase (MAPK) pathway. The pathway for this activation is not yet fully understood but it may involve activation of FAK.

1.8.2.2 Integrins and cancer

The role of integrins in cancer cells is intriguing. There are potentially many ways in which these molecules may contribute to the suppression or promotion of the malignant phenotype. This is evident from the knowledge of their role in normal cells of cell survival and proliferation, differentiation and cell signalling. Integrin expression in normal tissues varies with tissue type. Because of the number of different integrins and their varying ligand binding specificity and affinities, it is difficult to generalise about the function of integrins in cancer cells. The role of integrins in cancer cells will also vary depending on the stage of the cancer. At different stages of progression the loss or gain of integrin expression may be advantageous to the tumour. Tumour progression and metastasis is a complex process, which requires cells to have different phenotypes at different times in the process. To be able to detach and migrate from the site of the primary tumour, cells must possess reduced adhesive interactions with surrounding cells and the extracellular matrix. When they reach their new location they must then be able to reattach and invade the surrounding tissue. One can envisage how loss and gain of integrin function at varying stages of tumour progression would provide these phenotypes. Many studies have shown a general down regulation of integrins in tumour

as compared to normal tissue including tumours of the endometrium, lung, colon, prostate and breast (Lessey et al. 1995), (Damjanovich et al. 1992) (Buck et al. 1990), (Hashida et al. 2001), (Perlino et al. 2000), (Alford and Taylor-Papadimitriou 1996). Other studies document differences in integrin expression between primary tumours and their metastases. Correlation of integrin expression with clinicopathological features of the tumour in question has been examined and certainly in melanoma, integrin expression may be of prognostic value (Vihinen et al. 2000). In metastatic melanomas, tumours that were beta1 positive rather than to beta1 negative had significantly longer disease free survival and the independent nature of beta1 integrin expression as a significant prognostic factor for survival after therapy was confirmed using multivariate analysis.

Increased expression of integrins in tumours of the oesophagus (Tanaka et al. 2000), liver (Ozaki et al. 1998) breast (Shaw 1999) have been described as well as significantly elevated levels of integrins in the peritoneal metastases from gastric cancer (Matsuoka et al. 2000).

1.8.2.3 Integrins and ovarian cancer

With respect to ovarian carcinoma in particular, the beta1 integrin subunit was expressed in 4 out of 4 cell lines and all of the nine primary ovarian tumours sampled and was associated with various different alpha subunits (Cannistra et al. 1995). In contrast to studies of malignant melanoma where alphavbeta3 is upregulated in association with the acquisition of the invasive phenotype, downregulation of beta3in ovarian cancer appears to be associated with the acquisition of invasive properties (Carreiras et al. 1996).

Alphavbeta3 is present in well-differentiated and non-invasive borderline tumours but is absent in invasive carcinomas.

1.8.3 Selectins

The selectin family of cell adhesion receptors is composed of 3 members; L, E and P selectin. They are composed of an amino-terminal domain that is homologous to calcium- dependent animal lectins, followed by an epidermal growth factor (EGF) type domain, two to nine complement regulatory protein repeats, a transmembrane segment and a short cytoplasmic tail. Selectins mediate heterotypic cell to cell interactions through calcium-dependent recognition of sialyated glycans. Selectins are only expressed in endothelial and blood cells. They promote leucocyte adherence to endothelial cells and platelets during inflammatory processes.

1.8.4 Immunoglobulin superfamily of cell adhesion molecules

1.8.4.1 Immunoglobulin superfamily of cell adhesion molecules in normal tissues

Cell adhesion molecules of the immunoglobulin superfamily of proteins mediate calcium -independent cell to cell adhesion. They are characterised by the presence of one or more immunoglobulin-like domains containing approximately 100 amino acid residues per domain. The immunoglobulin-like domains are homologous to those found in antibody molecules. These domains are identified at the primary sequence level by the presence of two cysteine residues separated by 55 to 75 amino acids (which form a disulphide bond in the folded structure) and a so called "invariant " tryptophan residue located 10-15 residues C terminal to the first conserved cysteine. In addition,

immunoglobulin cell adhesion molecules contain varying numbers of fibronectin type III domains. Subfamilies of the immunoglobulin cell adhesion molecules are grouped based on the respective number of each type of domain, the mode of attachment to the cell membrane and the presence of a catalytic cytoplasmic domain.

Neural cell adhesion molecule (N-CAM) is the most prevalent immunoglobulin cell adhesion molecule in vertebrates and like cadherins, it binds cells together by a homophilic interaction. Some immunoglobulin cell adhesion molecules such as the intercellular cell adhesion molecule subfamily (ICAMs) use a heterophilic mechanism of adhesion.

N-CAM is widely expressed in the nervous system and is also expressed in other tissues. There are many isoforms of N-CAM produced by alternative splicing of an RNA transcript from a single gene. N-CAM contains five immunoglobulin domains and two fibronectin type III domains. Most N-CAMs are single pass transmembrane proteins but one isoform does not cross the lipid bilayer and is attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. The three most common isoforms are the GPI-linked 120kDa isoform (NCAM120) and the 140kDa and 180kDa transmembrane forms (NCAM140 and NCAM180). Further variation in N-CAM arises as a result of post translational modification by the addition of negatively charged sialic acid residues. These modifications alter the adhesive properties of N-CAM. In the nervous system, N-CAM plays a role in neurite outgrowth and guidance. It is the prototype for the large family of neural cell adhesion molecules of the immunoglobulin superfamily. As such, the induction of neurite outgrowth has been intensively studied in an attempt to elucidate the signalling mechanisms induced following binding of this cell adhesion molecule.

The original assay devised by Doherty and Walsh (Doherty et al. 1989) was to grow PC12 cells or primary neurons on supporting cell monolayers or fibroblasts that had been transfected with various isoforms of adhesion molecules. Untransfected cells were used to control for levels of neurite outgrowth not dependent on the transfected cell adhesion molecules. Cell adhesion molecules including N-CAM, L1 and N-cadherin promoted neurite outgrowth by two to threefold over that seen on untransfected monolayers. For N-CAM, both transmembrane and GPI- linked forms in the transfected supporting cell layer supported neurite extension suggesting that an extracellular portion of N-CAM was important for signal transduction. To determine intracellular signals important for cell adhesion molecule enhanced neurite outgrowth, the extent of neurite outgrowth was measured in the presence of agents that enhance or block the activity of particular second messenger systems. These experiments showed that cell adhesion molecule dependent neurite outgrowth was dependent on G protein stimulated activation of calcium channels (Doherty et al. 1991). Specific tyrosine kinases have been implicated in the effects of cell adhesion molecules on neurite outgrowth. Inhibiting receptor tyrosine kinases with an analogue, erbstatin, blocked the ability of N-CAM and L1 to stimulate neurite outgrowth (Williams, Walsh, and Doherty 1994) suggesting that a tyrosine kinase is an upstream regulator of the G proteins and calcium channels that were proposed to mediate neurite outgrowth. This led to a hypothesis involving the fibroblast growth factor receptor (FGF-R). This hypothesis proposed that the effects of cell adhesion molecules on neurite outgrowth could be attributable to their ability to stimulate the FGF-R (Williams et al. 1994) (Williams et al. 1995). The authors identified a region in the FGF-R that contained sequences similar to short peptide regions within

the extracellular regions of the N-CAM, L1 and N- cadherin proteins. This observation led to the suggestion that this "cell adhesion molecule homology domain" in the FGF-R was involved in interactions with the cell adhesion molecule proteins. Evidence for a direct interaction between N-CAM and the FGF-R has now been obtained (Cavallaro et al. 2001). In this paper the author showed that N-CAM modulates pancreatic tumour cell adhesion to matrix by inducing FGF-R signalling.

1.8.4.2 Immunoglobulin superfamily of cell adhesion molecules and cancer

In many human cancers, including Wilm's tumour, colon carcinoma, Ewing's sarcoma, neuroblastoma, small cell lung carcinoma and multiple myeloma, NCAM expression changes from NCAM120 in the normal tissue to NCAM140 and NCAM180 in tumours. In addition, in human pancreatic and colorectal carcinomas reduced levels of NCAM expression correlate with increased tumour malignancy (Fogar et al. 1997) More recently, loss of NCAM expression has been correlated with progression to tumour metastasis (Perl et al. 1999).

The DCC family of immunoglobulin cell adhesion molecules is a subfamily containing four immunoglobulin domains and six fibronectin type III repeats. The most prominent member is Deleted in Colon cancer (DCC) which was originally identified as a putative tumour suppressor gene from the 18q region of frequent LOH (Fearon et al. 1990). DCC is a receptor for the laminin–related netrin1 extracellular matrix protein. In normal tissues, abundant expression of DCC is found in the central and peripheral nervous system as well as most epithelial tissues (Hedrick et al. 1994) (Reale et al. 1994) Expression of DCC is reduced or absent in a variety of human malignancies. The

function of DCC in normal and cancer tissues is however still relatively poorly understood. Mice lacking DCC show defects in axonal projections but growth, differentiation, morphogenesis and also tumorigenesis in mouse intestine are unaffected (Fazeli et al. 1997). DCC is expressed in normal human ovarian surface epithelium. In the largest study to date of DCC in ovarian carcinoma, significantly decreased levels of overall DCC values in carcinomas compared with benign and low malignant potential lesions were revealed by both immunohistochemical and RT-PCR-Southern blot hybridisation assays. In carcinomas, reduction or loss of DCC expression was significantly related to the serous phenotype, high histological grade, and a more advanced stage. There was no association with survival (Saegusa, Machida, and Okayasu 2000). In ovarian carcinomas therefore, it appears that altered expression of the cell adhesion molecule DCC is closely linked with tumour differentiation and progression.

1.8.4.3 IgLON family of cell adhesion molecules

The IgLONS are also members of the immunoglobulin super family of proteins. They are characterised by the presence of three immunoglobulin-like domains and are extracellular molecules that are linked to the plasma membrane by a glycosyl phosphatidyl inositol (GPI) anchor. There are four family members; opioid binding cell adhesion molecule (OBCAM) (Schofield et al. 1989), neurotrimin (CEPU) (NTM) (Struyk et al. 1995) limbic system-associated protein (LAMP) (Pimenta, Fischer, and Levitt 1996) and kilon (neurotractin) (Funatsu et al. 1999).

All four of the IgLONs are expressed widely in the central nervous system during development and adulthood. Almost all of the work done on these molecules has investigated their role in neurodevelopmental biology. As these proteins are cell surface molecules, extensive research has been done investigating the nature of interactions that these proteins make. LAMP, CEPU and OBCAM have been shown to interact homophilically and heterophilically with each other (Lodge et al. 2000). LAMP and CEPU also interact with neurotractin but Neurotractin does not appear to bind homophilically (Marg et al. 1999). NTM-Fc binds to neurons that have had GPI linked proteins removed by phospholipase C treatment (Gil et al. 1998). This suggests that the IgLONs form heterophilic interactions with molecules other than IgLONS.

In addition to these trans interactions, IgLONs also form cis interactions. Homodimers and trimers of NTM have been identified in the plane of the cell membrane (Gil et al. 1998).

1.9 GPI-ANCHORED MOLECULES

Diverse arrays of proteins are attached to the cell surface by GPI anchors. These include proteins that regulate the activation of the complement cascade (eg DAF1), control T-cell activation and affect neural cell adhesion (an isoform of NCAM). Despite the varied functions of these proteins they are all attached to the cell membrane by a common glycolipid anchor. The anchor is composed of phosphatidyl inositol (spans the external leaflet of the membrane), glucosamine, three mannoses and a phosphorylethanolamine (Yeh, Kamitani, and Chang 1994). Proteins destined to be GPI linked have both N and C terminal signal peptides to which the preformed GPI anchor is attached within the

endoplasmic reticulum. Our understanding of how GPI anchored molecules transduce signals is far from complete but there are a few lines of evidence to suggest how signalling may be accomplished.

GPI anchored proteins have been shown to be part of functional rafts within the cell membrane. Rafts are formed by the association of cholesterol and sphingolipids and can be isolated as detergent insoluble complexes following treatment with the detergent Triton –x (Brown and Rose 1992). Clustering of GPI anchored proteins in rafts may allow cis interactions with signal transducing transmembrane proteins. Signal transduction molecules such as the src family of non receptor tyrosine kinases and heterotrimeric G proteins have been detected in the detergent insoluble complexes after Triton-x treatment and have been shown to co-immunoprecipitate with GPI anchored proteins in haemopoetic cells.

1.10 OBJECTIVES OF RESEARCH

The starting point for the work in this thesis was the finding of several independent studies showing a high frequency of LOH in the 11q24-5 region in epithelial ovarian cancer. (Foulkes et al. 1993) (Gabra et al. 1995) (Gabra et al. 1996) (Davis et al. 1996). These studies suggested that this region houses a tumour suppressor gene involved in this disease. The initial period of work was to entail an LOH analysis of all sixteen polymorphic microsatellite markers from the region in a panel of DNAs derived from 112 patients with epithelial ovarian cancer. The intention was then to identify candidate tumour suppressor genes near markers with interesting LOH findings. Having identified candidate genes the plan was to look for evidence of inactivation of these genes in

ovarian cancer cell lines and primary ovarian tumours. Transfection of candidate genes were then to be performed to allow functional studies to be undertaken.

The most striking finding of the LOH analysis was a high frequency of LOH at the marker D11S4085 and this marker was found to lie within an intron of a gene called OBCAM. OBCAM therefore became a candidate for the tumour suppressor gene in this region and much of the work in this thesis relates to the investigation of the potential role of OBCAM as a tumour suppressor gene in ovarian cancer. Expression studies of OBCAM in normal tissues, tumours and cell lines were undertaken. Having identified LOH as a "first hit "of this gene in ovarian cancer, search for a "second hit" in the form of CpG island methylation and mutation screening was performed. Transfection studies allowed development of cell lines that were used in *in vitro* and *in vivo* assays.

Additionally, analysis of several other potential tumour suppressor genes from the region was undertaken and this data is also reported.

2. METHODS AND MATERIALS

2.1 Mapping and bioinformatics

At the start of the project in August 1999, 16 polymorphic microsatellite markers from the 11q24-25 region were identified from a radiation hybrid map of chromosome 11.The majority of the microsatellite sequences and the corresponding primer pairs were available from the GenBank sequence database. Two sequences (1884 and 1895) were determined by sequencing. Initially, physical mapping using YACs from the 11q24 region was undertaken to allow markers and known genes to be ordered with respect to each other. This produced a crude map of the region.

To refine this map and confirm the order of the microsatellite markers and identify genes within this region a BAC contig tiling path was created. To do this, each microsatellite sequence was put into BLAST (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov) and searched against the HTGS database. BLAST is a program that aligns the sequence of interest with a specified database. BACS on chromosome 11 that contained the microsatellite sequence were then selected. These BACS were then subjected to NIX analysis. NIX (Nucleotide identify X) (http://www.hgmp.mrc.ac.uk/NIX/) is a tool to aid the identification of interesting regions in genomic or transcribed nucleic acid sequences. Although many individual computer prediction programs have been developed to do such analysis, these programs all have strengths and weaknesses. NIX integrates many of these programs and displays the results side by side. Such direct comparison allows us to identify when several programs have a consensus feature that strengthens the likelihood of the prediction being correct.

Initially the NIX program was used on a very simple level to pick out the microsatellite marker and position of related genes. At this stage the human genome project was in its early stages for this region. At the end of 1999 several BACS for most microsatellite markers had been identified. The BACS were all at differing levels of sequence completion, often being composed of more than 20 separate contigs. The presence of unordered contigs within the BACS meant that there was always uncertainty with the map. It was continually updated as new information was released from the Human genome project.

2.2 LOH Analysis

2.2.1 Clinical material

A panel of 112 matched normal/tumour DNA samples from patients with epithelial ovarian cancer was assembled. 49 of the DNA pairs were made from paraffin embedded normal and tumour tissue blocks obtained from the Department of Pathology, Edinburgh University. A further 38 pairs were made from fresh tissue collected from the Department of Gynaecology, Edinburgh Royal Infirmary. The remaining 25 pairs were a gift from Ian Brown (University of Aberdeen). All clinical material was obtained following patient consent and the work carried out in conformity with current ethical approval.

2.2.2 Oligonucleotide primers

Fluorescently labelled oligonucleotide primers for sixteen polymorphic microsatellite markers from the 11q24-25 region were obtained from the ICRF oligonucleotide laboratory at Clare Hall.

Table 2.1 Chromosome 11q24-5 microsatellite markers used in LOH analysis

Microsatellite	Sequence 5'-3'	~ size	Dye
D11S1894	**FOR** AGATCCCTACAGAACTCTCTCTACA	116	FAM
	REV TAGAGTAAGTGTCATGGGGTGTC		
D11S912	**FOR** TGCTTTGGGTATGCATATGT	110	FAM
	REV AGCTTTTTGTCTAGCCATGA		
D11S4150	**FOR** TCTCCCTGTTGTGGGG	250	FAM
	REV TTACTACCCAGAGATTCATTTGT		
GATA69G01	**FOR** AATTAGAGATGTAGGTGTGAACTCA	84	FAM
	REV TATATCTACATTGCAGCTAGGTAGG		
D11S4126	**FOR** TTGCAGTGAGACAAAGCAAG	100	FAM
	REV GTCAGAAGCCTGTGCCG		
D11S4131	**FOR** TCAAGAGCAGTGGTCTAGNTCAAGA	100	FAM
	REV ATCACTAGCCAAAGCAGCAG		
GATA72A01	**FOR** TAGGAATATCCAGGCATTGC	150	TET
	REV CAGAGGACTTGGAAGCACTT		
D11S1884	**FOR** GGTCAGACCCAAGACTTCA	155	FAM
	REV TGGCTCACCAAGGATTTA		
D11S910	**FOR** AGCTTTGCAGACAAGGCAAG	156	TET
	REV2 TCTAAGGCATGACATTTCAC		
D11S1320	**FOR** AACATTACTAAAAGGTTAAATGAGC	230	TET
	REV ATTAAGGCACCAAATGGG		
D11S874	**FOR** AATCATTTTCAAGCATAGGC	160	FAM
	REV GGTTTAAAAAGTCAGCCCTC		
D11S4085	**FOR** GCTACAATATCAATAGAAGG	200	FAM
	REV GGCCACAGGACTTTCAGAG		
D11S969	**FOR** TTGATTTGGAAGATTTTCAC	145	FAM
	REV GGGGCAGAATGGGTAT		
D11S1309	**FOR** CCCTTCGTCACAGAAACA	240	FAM
	REV ACTCTAGCCCACAAATTAAGACT		
D11S4198	**FOR** ACTTGGACCGAATGCTGT	120	TET
	REV GCTAATCCAGAAGACTTGCC		
D11S1895	**FOR2** CAGGGCATTCAATCCTTA	136	TET
	REV2 TGCTACTTTGAGGATGTC		

2.2.3 Treatment of oligonucleotides

The primers were supplied fully deprotected and dried down. Ethanol precipitation was used to remove the side products of the synthesis. Each oligonucleotide was dissolved in 200µl containing 20µl 3M sodium acetate (pH5.6), 20µl 100mM magnesium chloride and 160µl DEPC water. 600µl of 100% ethanol was added and mixed by briefly vortexing. The samples were left at –70C for at least 30 minutes. Samples were then centrifuged at 13000rpm for 20 minutes at 4^0C and the supernatant discarded. The pellet was washed with 250µl 70% ethanol in DEPC water and spun at 13000rpm for 5 minutes at 4C. The supernatant was removed and the pellet dried at room temperature. The dry pellet was resuspended in 200µl of DEPC water. 5µl of primer solution was added to 995µl DEPC water. The OD260 of the 1:200 dilution was measured on a spectrophotometer and the concentration of primer solution determined using the following formula;

$$\text{Stock concentration} = \frac{\text{OD260 x dilution (200) x } 1000}{\text{Extinction coefficient}} = \text{pmol/µl} = \text{µM}$$

2.2.4 Optimisation of PCR conditions

The reaction volume for each PCR was 25µl. Each PCR contained 2.5µl 10x buffer, 0.5µl 10mM dNTP, 0.8µl ICRF Taq polymerase and 0.25µl each of the forward and reverse primers (50µM stock). The optimal magnesium concentration for each primer pair differed and is shown below. 1µl DNA was added to each reaction and the volume made up to 25µl with water. A touchdown program from 67-55^0C was used as follows:

1.94^{0}C for 5 mins, 2. 67^{0}C for 30secs –3^{0}C per cycle, 3.72^{0}C for 45secs, 4.Go to 2, 3 times, 5.94^{0}C for 30secs, 6.55^{0}C for 30secs, 7.72^{0}C for 45secs, 8. Go to 6, 29 times, 9.72^{0}C for 5mins, 10.16^{0}C forever, end.

2.2.5 Checking of PCR products by agarose gel electrophoresis

8µl of PCR product was mixed with 2µl of glycerol loading dye and run on a 2% agarose gel containing ethidium bromide at 120 volts in 1x TAE. A 100bp DNA ladder was also run to confirm the size of the products. The products were visualised under UV light.

2.2.6 Allelotype analysis

Dilutions of PCR products containing the size standard Tamra 350 and deionised formamide were prepared and denatured for running on the 310 ABI prism.

Allelotype analysis was performed using the genescan software. Each pair of samples was assigned to one of the following groups; heterozygous, uninformative (homozygous) or nondeterminable. For the heterozygotes, the relative ratios of alleles were determined. LOH was assigned where the tumour allele ratio differed from the normal allele ratio by greater than 30% ($r < 0.7$).

2.3 Gene expression in normal ovary, normal tissues, cell lines and tumour samples

To determine the expression of candidate genes in normal ovary, cell lines and tumour samples RNA was first extracted from the respective samples. The RNA was DNAse1 treated and first strand cDNA synthesis performed.

2.3.1 RNA extraction from cell lines using Tri Reagent

Cell lines were grown in 175cm^3 until 70% confluent. The media was removed and 17.5ml Tri Reagent (1ml per 10cm^2 culture plate surface area) added to the flask to detach and lyse the cells. After addition of the Tri Reagent, the cells were repeatedly pipetted to form a homogenous lysate and the sample split equally into two 15ml Sarstedt polypropylene tubes. At this stage samples can be frozen at –70^{0}C for up to 1 month.

If the samples had been frozen they were thawed and incubated at room temperature for 5 minutes to ensure dissociation of nucleoprotein complexes. 1.7ml chloroform was added per tube using an RNase free glass pipette. The sample was covered and shaken vigorously for 15 seconds. Following a 10 minute incubation at room temperature the samples were centrifuged at 12000g for 15 minutes at 4^{0}Cin the SorvalRC5B centrifuge using the SS34 rotor. This separated the mixture into 3 layers. To precipitate the RNA, the upper aqueous phase was transferred into a fresh Sarstedt tube using a sterile pastette. 4.25ml isopropanol was added per tube and the samples inverted to mix. After a further 10 minute incubation at room temperature the samples were spun at 12000g for 10 minutes at 4^{0}C. An RNA pellet was visible at this stage. To wash the RNA, the supernatant was discarded. 10ml 75% ethanol in DEPC water was added and mixed by

vortexing. After a 5 minute centrifuge at 12000g at 4^0C the supernatant was removed and discarded. 0.5ml 75% ethanol in DEPC water was used to transfer and combine the pellets in a sterile microfuge tube. The tubes were washed out with a further 5ml 75% ethanol in DEPC water. The samples were spun in a microfuge at 13000rpm for 5 minutes at 4^0C. To solubilise the RNA, the ethanol was removed and the sample allowed to air dry for 15 minutes in a class II hood. 100µl DEPC water was added and the sample pipetted to mix. The RNA was then incubated for 15 minutes at 55^0C. The sample was placed on ice and quantitation of RNA performed using a spectrophotometer. The RNA was stored as 20µg aliquots at -70^0C.

2.3.2 Isolation of Total RNA from Normal Ovary

Human normal ovary (100 mg), snap frozen in liquid nitrogen at time of collection, was pulverised in a Mikro-Dismembrator U (B.Braun Biotech International) for 2 minutes at 2,000rpm. 1ml of Tri Reagent (Sigma) was then added and the protocol for RNA extraction from cell lines followed with all reagent volumes scaled down accordingly. RNA from the primary human ovarian tumours had been made previously.

2.3.3 DNase1 treatment of RNA

This procedure is used to remove contaminating DNA from the RNA sample.

5.7µl 10x buffer and 1µl Dnase1 (10units/µl)(Message Clean Kit) were added to 20µg of RNA and the volume made up to 57µl with DEPC water. This was mixed well and incubated for 30 minutes at 37^0C. A phenol/chloroform extraction was then undertaken to ensure removal of protein contamination and DNase1 from the RNA. 30µl phenol and

10µl chloroform were added and vortexed for 30 seconds. Following a 10 minute incubation on ice the sample was spun at 13000rpm for 5 minutes at 4^0C. The upper phase was removed into a 200µl microfuge tube. An ethanol precipitation was then performed. 5µl 3M sodium acetate and 200µl 100% ethanol were added and mixed by a brief vortex. The sample was incubated at -70^0C for more than an hour and then spun at 13000rpm for 10 minutes at 4^0C. The supernatant was carefully removed and discarded and the RNA pellet washed with 500µl 70% ethanol in DEPC water. After a brief vortex the sample was spun at 13000rpm for 5 minutes at 4^0C and the supernatant discarded. The RNA was dissolved in 10µl DEPC water and 1µl used to make a 1:1000 dilution for quantitation on the spectrophotometer. The DNase treated RNA was stored as 1µg aliquots at -70^0C.

2.3.4 First strand cDNA synthesis

Using the first strand cDNA synthesis kit (Boehringer Mannheim), RNA is reverse transcribed into single stranded cDNA. In this method, using the oligo p(dT) primer, AMV reverse transcriptase synthesises the new cDNA strand at the 3' end of the poly (A) mRNA. The resulting first strand cDNA can then be used as a template for PCR.

1µg of RNA was used for each first strand cDNA synthesis of 20µl. A master mix of reagents was prepared and a varying volume of water added to each RNA to give a final reaction volume of 20µl. To degrade the secondary structure of the RNA, the RNA/water mixture was denatured at 65^0C for 15 minutes and then placed on ice for 5 minutes.

A master mix of reagents was prepared to include 2µl 10X buffer, 4µl 25mM $MgCl_2$, 2µl deoxynucleotide mix, 2µl oligo p(dT) primer and 1µl RNAse inhibitor per reaction. The master mix was divided equally into two tubes and 0.8µl AMV reverse transcriptase added to the first tube. This allowed a first strand cDNA synthesis (+RT) and a control reaction (-RT) to be produced for each RNA sample. (2x1µg aliquots RNA required). Each reaction was incubated at 42^0C for 60 minutes for reverse transcription to occur, followed by a 5 minute incubation at 95^0C to denature the AMV reverse transcriptase. The samples were cooled on ice and aliquots of 2µl first strand cDNA prepared and frozen at -70^0C.

2.3.5 Quantitative determination of gene expression using a Light Cycler

The level of OBCAM expression was determined by quantitative RT-PCR using a Light Cycler (Roche) (Euan Stronach) in a series of 18 ovarian primary tumours (DNaseI-treated total RNAs were a gift from Dr. Simon Langdon) and 17 ovarian and 14 non ovarian cancer cell lines. Quantitation of PCR products was performed via detection of incorporated SYBR Green I. OBCAM expression was calculated relative to Actin expression in each sample, and then expressed as a percentage normalised to the expression level in a sample of normal ovary.

Table 2.2 Primers for the determination of OBCAM expression by Light Cycler

Primer	Sequence 5'-3'	Acc. No	Nucleotide	Size bp
OBCAM FOR4	TACCATAGATGACCGGGTAA	NM_002545.2	221-240	224
OBCAM REV4	CTATTAGGTGAACCCGGGAC	NM_002545.2	444-425	
Beta Actin FOR	CTACGTCGCCCTGGACTTCGAGC	NM_001101	724-746	385
Beta Actin REV	GATGGAGCCGCCGATCCACACGG	NM_001101	1108-1086	

2.3.6 Semi-quantitative determination of gene expression using RT-PCR

Multiple Tissue cDNA (MTC) panels (Human I and Human II; BD Biosciences, Basingstoke, UK) were tested for OBCAM expression by semi-quantitative RT-PCR using the Advantage 2 PCR kit (BD Biosciences) with an OBCAM primer pair to identify the full length OBCAM transcript (Genevieve Rabiasz)

Table 2.3 Primers for the determination of OBCAM expression by semiquantitative RT-PCR

Primer	Sequence 5'-3'	Acc. No	Nucleotide	Size bp
OPCML FOR1	AGTTGTGGCTGTCGAGAATG	NM_002454.2	34-53	1079
OPCML REV1	TCAGAGGACCTAGGATTTCT	NM_002454.2	1110-1091	

Semi-quantitative RT-PCR was also used to determine Fli1 gene expression in a panel of thirty human ovarian tumours (HOVs) and thirteen ovarian cancer cell lines.

Table 2.4 Primers for the determination of Fli1 expression by semi-quantitative RT-PCR

Primer	Sequence 5'-3'	Acc. No	Nucleotide	Size bp
Fli1 9BFOR	AACGCCAGCTGTATCACCTG	NM_002017.2	1058-1077	301
Fli1 9CREV	GGGACAAAGTTCACCTTCTG	NM_002017.2	1377-1358	

2.4 Mutation Detection by Single Strand Conformation Polymorphism Electrophoresis (SSCPE)

Single Strand Conformation Polymorphism Electrophoresis (SSCPE) is a technique used to identify abnormalities in nucleotide sequence. A single nucleotide difference between two short single stranded DNA molecules induces a difference in the conformations adopted by the two strands. This conformational change is sufficient to produce changes in the molecules' electrophoretic mobilities on neutral polyacrylamide gels. The difference in electrophoretic mobility is seen as a band shift on the gel.

SSCPE analysis was performed on the OBCAM, Fli1 and Barx2 genes. SSCPE of the OBCAM and Fli1 genes was performed by Diane Scott, Eric Miller and Charles Massie. SSCPE of the Barx2 was performed by Claire Taylor at the Cancer Research UK Mutation Detection Facility in Leeds. DNA samples used in the SSCPE analysis were extracted from fresh and archival ovarian tumour and matched normal tissues and ovarian and non ovarian cancer cell lines.

2.4.1 Primers for SSCPE

Bioinformatic comparison of OBCAM mRNA and Human Genome Project sequences predicted the human OBCAM gene to comprise 7 exons. PCR primers to amplify the predicted exons were designed (Grant Sellar). Two overlapping primer sets were required to span exon 2 due to size limitation of PCR product useful for SSCPE analysis. The OBCAM exon specific primers are documented in table 2.5 .

Table 2.5 Primers for OBCAM SSCPE

Exon	Primer	Sequence 5'-3'	Acc.No	Nucleotide	Size bp
1	OBCAM1FOR	GACCAGGACTGTGCGGCTGC	NM_002545.2	1-20	188
	OBCAM1REV	CGTCACGTTGTCCATAGCTT	NM_002545.2	188-169	
2	OBCAM2AFOR	CACCACTCCCTGCCTCACTG	AC012234.6	75226-75245	175
	OBCAM2AREV	CATCCACATTTTGGATCATG	AC012234.6	75400-75381	
	OBCAM2BFOR	ATAGACCCTCGTGTGATCAT	AC012234.6	75331-75350	180
	OBCAM2BREV	TGGCAACCCCAGATCCAGCT	AC012234.6	75510-75491	
3	OBCAM3FOR	CAGGTATTTCTTCTATCCTG	AP000843.3	37032-37051	179
	OBCAM3REV	GTCCTCCAGGTCAGCACCTT	AP000843.3	37210-37191	
4	OBCAM4FOR	TGGTTACACAGTTTCCTGAT	AP000843.3	2881-2900	214
	OBCAM4REV	AGAACCCCCTGGCTGCAGGT	AP000843.3	3094-3075	
5	OBCAM5FOR	GTGCGTGCATGCCTGTGCAT	AP000843.3	3466-3485	195
	OBCAM5REV	CAGAACTGTCCAGGTGTCAT	AP000843.3	3660-3641	
6	OBCAM6FOR	TAGCAATGTCTTCCCTCTTG	AP000843.3	4028-4047	198
	OBCAM6REV	GCATCCAGGCTTCCAGCACT	AP000843.3	4225-4206	
7	OBCAM7FOR	TCCTTGGGTGTATGCTAATG	AP000843.3	19945-19964	176
	OBCAM7REV	GCGTTGCTCAGAGGACCTAG	AP000843.3	20120-20101	

Primer pairs were designed for each of the 9 exons of Fli1, amplifying from flanking introns across the exons. Four overlapping SSCPE primer pairs for exon 9 were required to allow complete analysis of this exon. In order to design these primers, the Fli1 mRNA sequence (NM_002017.2) was blasted against BAC AP000920 that was known to contain sequence of the Fli1 gene. This allowed identification of the intronic sequence flanking the exons. The primers are shown in table 2.6.

Table 2.6 Primers for Fli1 SSCPE

Exon	Primer	Sequence 5'-3'	Acc.No	Nucleotides	Size bp
1	Fli1 1DFOR	CTGCGAGGTCAGGCTGTAAC	AF275879.1	4311-4330	191
	Fli1 1DREV	GGGAGAGAGGCCACGTCTTC	AF275879.1	4501-4482	
2	Fli1 2CFOR2	GTGAAGAGTGACACTGGGCT	AP000920	56384-56365	114
	Fli1 2CREV2	AGTCATGTCGGCCTTGGGGA	AP000920	56253-56272	
	Fli1 2DFOR	TCCCCAAGGCCGACATGACT	AP000920	56243-56224	125
	Fli1 2DREV	CAATCCTGCACAGGCCTGGT	AP000920	56100-56119	
3	Fli1 3AFOR	AGTGCTGACCGCCTCTGGGC	AP000920	9811-9830	219
	Fli1 3AREV	CAGGCAGCCTGGTTCTCGAA	AP000920	10029-10010	
4	Fli1 4CFOR	CAATGGCTGGAGTGGGCCAT	AP000920	14531-14550	194
	Fli1 4CREV	CTGGGTACTTGGGCGGCACT	AP000920	14725-14706	
5	Fli1 5AFOR	CCTACTCTTGATCTCAGAAG	AP000920	23617-23636	176
	Fli1 5AREV	GTCATGTCTGGTACAGAAGA	AP000920	23792-23773	
6	Fli1 6AF0R	CTACAGATAATGCTCATGCA	AP000920	46716-46735	195
	Fli1 6AREV	ATCCTGTCCTCAGAAACATG	AP000920	47210-47191	
7	Fli1 7FOR	AGCTGGGTCCTACTCACTGC	AP000920	63456-63475	175
	Fli1 7REV	TCCATCAGTTGACCATGTGA	AP000920	63630-63611	
8	Fli1 8FOR2	ACACTGAAGCAAATTTCCTT	AP000920	65439-65458	179
	Fli1 8REV2	CCACTCAGGTGTCTGGACTT	AP000920	65617-65598	
9	Fli1 9AFOR	CATTTAGGGAACTGGGTTCT	AP000920	66700-66720	179
	Fli1 9AREV	CATTTTGAACTCCCCGTTGG	AP000920	66878-66859	
	Fli1 9BFOR	AACGCCAGCTGTATCACCTG	AP000920	66831-66850	174
	Fli1 9BREV	GCCGTGCACTTTGGTCATAA	AP000920	67004-67985	
	Fli1 9CFOR	GGCCCTCCGTTATTACTATG	AP000920	66956-66975	171
	Fli1 9CREV	GGGACAAAGTTCACCTTCTG	AP000920	67126-67107	
	Fli1 9DFOR2	GTACCCTTCTGACATCTCCT	AP000920	67085-67104	164
	Fli1 9DREV2	TGGGGTTGGGGTAGATTCCC	AP000920	67248-67229	
	Fli1 9EFOR1	GCATCACAATACTGGACCTC	AP000920	67200-67219	172
	Fli1 9EREV1	TGAGTCCAAAGCATCCAGTA	AP000920	67371-67352	

Primers for SSCPE analysis of the 4 exon Barx2 gene were designed by Hani Gabra and are shown in table 2.7.

Table 2.7 Primers for Barx2 SSCPE

Exon	Primer	Sequence 5'-3'	Acc. No	Nucleotides	Size bp
1	BARX2F11B	ATGCACTGCCACGCCGAGCT	AF171219	1650-1669	145
	BARX2R11	ACACGGAGTAGAGGGAAAGT	AF171219	1770-1789	
	BARX2F1	ATGATCGACGAGATCCTCTC	AF171219	1723-1742	186
	BARX2R5	TACACGGACGTGAAAGCTAC	AF171219	1869-1888	
2	BARX2F6	CAGGTCCTGGCCTGCTTCCC	AF171220	76-95	165
	BARX2R12	AGGACAGTGCCTGGGCGATT	AF171220	228-247	
	BARX2F13	TCATCTCCCACCTGGTCCCT	AF171220	197-216	182
	BARX2R13	CTCGGTGAAGATGGTGCGAC	AF171220	359-378	
	BARX2F14	CGAGTCAGAGACGGAACACC	AF171220	309-328	202
	BARX2R6	CCCACAATGGGAGCAAGTCT	AF171220	491-510	
3	BARX2F10	TCCTGCTGCCTCCCATTCTG	AF171221	284-303	134
	BARX2R14	TCTTCCATTTCATCCTGCGA	AF171221	396-415	
	BARX2F15	TCAGTCTCTGGGACTCACTC	AF171221	348-367	167
	BARX2R15	CAAACTGCCAAATGGTCCGG	Unpublished	sequence	
4	BARX2F8	TGGAGGGAAGGAATTATTTC	AF171222	313-332	155
	BARX2R16	CTGTTCATCTTCTCTTCAGC	AF171222	449-468	
	BARX2F16	GAACTCCATCCCCACATCAG	AF171222	418-437	160
	BARX2R17	TGGTGGCTCTGCCATCTCTA	AF171222	558-577	
	BARX2F17	AGGAGGAGCTCTGTGAAGCA	AF171222	500-519	153
	BARX2R8	AGTCTCCCTCTTCCCTCAAA	AF171222	643-662	

PCR conditions for all primer pairs were optimised in 25µl reaction volumes. SSCPE and silver staining of 5-10µl denatured PCR product was carried out as described.

2.4.2 Electrophoresis

A non denaturing 10% acrylamide, 3% bis-acrylamide gel was prepared using 1xTBE.Depending on the strength of PCR product, either 5µl or 10µl was mixed with 2µl or 4µl denaturing dye respectively (95% formamide, 20mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol). This mixture was then denatured at 98^0C for 3 minutes and place immediately onto ice. The mix was loaded into the gel with

duckbilled tips and run at room temperature for 20 minutes at 20mA before transferring to the cold room (4^0C). The gel was run overnight for between 14-16 hours at 10mA per gel. The running buffer was 1xTBE.

2.4.3 Silver staining

Following electrophoresis, the gel was transferred gently into a Pyrex dish for silver staining. The gel was soaked in 10% ethanol for 5 minutes, 1% nitric acid for 3 minutes and then briefly rinsed in distilled water. The gel was soaked for 20 minutes in a freshly prepared solution of 1mg/ml silver nitrate in distilled water, ensuring that the silver nitrate had been completely dissolved before adding to the gel. The developer buffer was prepared by dissolving 15g of anhydrous sodium carbonate in 500ml distilled water and adding 250µl formaldehyde. The gel was rinsed in distilled water to remove the silver nitrate and 100ml of the developer buffer added until the buffer turned brown and was discarded. The remaining 400ml developer buffer was added and at this stage the nucleic acid bands became visible. When the desired intensity had been reached the developer buffer was discarded. The gel was rinsed twice in water and the reaction was stopped with 10% glacial acetic acid for 2 minutes. A photograph was taken of the gel after which it was dried on the gel drier at 85^0C for 3 hours to make a permanent record.

2.4.4 Exo/sap purification of PCR products

PCR products that produced band shifts were sequenced to detect any nucleotide alterations compared with the GenBank reference sequences. Prior to sequencing, the PCR products were purified using a combination of shrimp alkaline phosphatase and

exonucleaseI. For the exosap purification, 2-3 µl of PCR product (maximum 6.5µl) was mixed with 0.5 µl ExoI (10U/µl), 1µl SAP (1u/µl) and water to a final volume of 8 µl. Products were run on the SAP program as follows; 37^0C for 15 minutes, 80^0C for 15 minutes and 4^0C hold.

2.4.5 Sequencing

4µl of EXO/SAP product was used for each of the forward and reverse sequencing reactions. To the 4µl of EXO/SAP mixture was added 4µl Big Dye, 1µl sequencing primer (3.2µM stock) and water to a final volume of 20 µl. The sequencing programme that follows was run; 96^0C hold, 96^0C for 30 seconds, 50^0C for 15 seconds, 60^0C for 4 minutes for 24 cycles then 4^0C hold.

2.4.6 Precipitation of sequencing reactions

The sequencing reactions were precipitated using the following method. For each sequencing reaction a 1.5 ml microfuge tube was prepared containing 2µl 3M sodium acetate (pH 4.6), 0.5µl pellet paint and 50µl of 100% ethanol. The contents of each tube were vortexed and left at room temperature for at least 15 minutes to precipitate the extension products. The tubes were then spun for 20 minutes at 13000 rpm at 4^0C. The supernatant was aspirated and discarded. The pellets were rinsed by the addition of 250µl of 70% ethanol and a brief vortex. The tubes were spun for 5 minutes at 13000rpm at 4^0C. The ethanol was removed using a fine tip pastette and the pellets allowed to dry briefly at room temperature. The precipitated sequences were run on an

ABI 377 DNA sequencer (Agnes Gallagher, Medical Research Council, Human Genetics Unit.).

2.5 Investigation of OBCAM homology between species and other human IgLON family members

Amino acid and nucleotide sequences for human OBCAM (HS), rat (RN), bovine (BT), chicken (GG), Drosophila (DM) and Caenorhabditis elegans (CE) OBCAM, were obtained from GenBank (http://www.ncbi.nlm.nih.gov), FlyBase (http://flybase.bio.indiana.edu/) and Worm Base (http://www.wormbase.org/) respectively. Sequences for human NTM and LAMP were also obtained from GenBank. Table 2.8 documents the accession number for each sequence. Investigation of OBCAM homology between species and the other human IgLON family members was performed using the Clustalx1.8 and LALIGN (http://www.ch.embnet.org/software/LALIGN_form.html) multiple sequence alignment programs.

Table 2.8 Accession numbers for OBCAM species and other IgLON family members

Species/IGLON	Accession Number
HS OBCAM	NM_002545.2
RN OBCAM	NM_053848.1
BT OBCAM	X12672.1
GG OBCAM	AF292934.1
DM OBCAM	CG11320
CE OBCAM	F41D9.3
LAMP	NM_002338.1
NTM	NM_016522.1

2.6 Methylation Specific PCR

Methylation specific PCR (MSP) is used to detect the presence or absence of methylation in DNA sequences. The technique consists of chemical modification followed by amplification of DNA. In bisulphite modification of the DNA, all unmethylated cytosines are converted to uracils, while 5-methyl cytosines remain unaltered. Thus, the sequence of the treated DNA will differ depending on whether the DNA was originally methylated or unmethylated. Primers are designed to specifically amplify the sequences based upon the chemically induced differences. PCR products can be further analysed on a gel or by DNA sequencing.

2.6.1 Methylation Specific PCR Assay

Genomic DNAs isolated from the normal/ovarian tumour matched pairs), cancer cell lines (ovarian, breast, lung, colon and pancreatic cancer), normal ovary, and control methylated DNA (CpGenome Universal Methylated DNA, Intergen, Purchase, NY, USA) were incubated with sodium bisulphite to deaminate unmethylated cytosines using the CpGenome Modification kit (Intergen). All MSP primer sequences were designed by Dr Hani Gabra with the exception of the Barx2 MSP primers.

For the OBCAM MSP assay, bisulphite-treated DNAs (40ng) from 'fresh' normal/ovarian tumour matched pairs, cell lines, and normal ovary, were PCR amplified in a 1-step assay with primer pairs to discriminate between the methylated (M) and unmethylated (U) alleles, respectively, of the human OBCAM CpG island. The 25 µl PCR reaction consisted of 1 x Buffer II (Applied Biosystems), 2.5 mM $MgCl_2$, 200 mM dNTPs, 200 nM each primer and 1 unit of AmpliTaq Gold DNA polymerase (Applied

Biosystems). PCR cycle conditions consisted of a hot start at 95 °C for 12 minutes, then 35 cycles of 95 °C for 30 s, (55 °C OBCAM3B-U) (56 °C U OBCAM1 / U OBCAM3B) (60 °C M OBCAM3B / M OBCAM1) for 30 s, 72 °C for 30 s and a final extension at 72 °C for 4 minutes. 10 µl of MSP product was then visualised on a 2.5 % agarose gel. A similar one step assay was used for MSP of the FLI1 and Barx2 genes (Gen Rabiasz).

For OBCAM, a two stage nested MSP assay was used with DNA extracted from archival specimens. The external primers, (OB2ST) amplify a 243bp PCR product from bisulphite modified DNA independent of methylation status. Modified DNA was initially amplified in a 25µl PCR reaction with the external primers. PCR products were then diluted 1/50 with distilled water and 2µl used in the second-step PCR using the primer combinations as detailed above for the 1-step assay (Gen Rabiasz). In the tables of primers used in the MSP assay, M = methylation specific primer, U= unmethylated specific primer.

Table 2.9 Primers for OBCAM MSP

Primer	**Sequence 5'-3'**	Accession Number	Nucleotides	Product size
M OBCAM IFOR	AGGCGTTTAGTGGAGGGGTACGGGC	NT_030800.5	824881-824905	136bp
M OBCAM1REV	CATCGCGCTACAATCGACTCCCCG	NT_030800.5	825016-824993	
U OBCAM 1FOR	AGGTGTTTAGTGGAGGGGTATGGGT	NT_030800.5	824881-824905	136bp
U OBCAM 1REV	CATCACACTACAATCAACTCCCCA	NT_030800.5	825016-824993	
M OBCAM 3BFOR	GCGCGGTGCGGGTTTATTTTC	NT_030800.5	825275-825295	135bp
M OBCAM 3REV	TCCCGATACCGCCTCGAAACGAACG	NT_030800.5	825409-825385	
U OBCAM 3BFOR	TCCCGATACCGCCTCGAAACGAACG	NT_030800.5	825275-825295	135bp
U OBCAM 3REV	GTGTGGTGTGGGTTTATTTTT	NT_030800.5	825409-825385	
OBCAM 2ST FOR	GTTTTTTTTGTAGGGGAAGT	NT_030800.5	825439-825419	244bp
OBCAM 2ST REV	CAACAACTCCATCCCTAACC	NT_030800.5	825196-825215	

Table 2.10 Primers for Fli1 MSP

Primer	Sequence 5'-3'	Accession Number	Nucleotides	Product size
M Fli1 BFOR	CGGTTCGTGGATTTTAAGTTAGGGC	AP001535.2	176453-176477	194
M Fli1 BREV	ACACGCTATACGCCAACCG	AP001535.2	176664-176646	
U Fli1 BFOR	TGGTTTGTGGATTTTAAGTTAGGGT	AP001535.2	176453-176477	194
U Fli1 BREV	ACACACTATACACCAACCA	AP001535.2	176664-176646	

Table 2.11 Primers for Barx2 MSP

Primer	Sequence 5'-3'	Accession Number	Product size
M BARX2 FOR	GGGATAACGTAGTTGTTAATTAGCGC	AF171219.1	194
M BARX2 REV	CAAAAACCGCCGAACACTAAACGCG	AF171219.1	
U BARX2 FOR	TTTGGGATAATGTAGTTGTTAATTAGTGT	AF171219.1	196
U BARX2 REV	CAACAAAAACCACCAAACACTAAACACA	AF171219.1	

2.6.2 Bisulphite Sequencing of the OBCAM CpG Island

MSP bisulphite-treated ovarian tumour and normal ovary DNA 529 bp products obtained by amplifying between U OBCAM1 / U OBCAM3B or U OBCAM1 / U OBCAM3B- were subcloned into the pGEM-T Easy TA cloning vector (Promega, Southampton, UK). Two to six subclones corresponding to each PCR product were then sequenced using Big Dye (PE Biosystems) chemistry following standard methods (Gen Rabiasz).

2.7 OBCAM Transfection into SKOV3

2.7.1 Preparation of plasmid DNA for transfection

The full coding sequence of human OBCAM plus Kozak consensus sequence and 3'UTR overlap encompassing nucleotides 34-1110 (NM_002454.2) was PCR amplified from normal human ovarian surface epithelium RNA with the primers OPCML F1 and

OPCML R1 primers previously tabled. The OBCAM PCR product was then subcloned into the pcDNA3.1 Zeo (zeocin-resistant) mammalian expression vector (In vitrogen) in both the sense and antisense orientations, and the insert sequence verified (Grant Sellar). Plasmid DNAs corresponding to OBCAM sense and antisense constructs were prepared using the QIAGEN plasmid purification kit. Prior to use in transfection experiments, the plasmid DNA was linearised with the enzyme PvuI. PvuI-linearised sense and antisense OBCAM constructs (2 μg) were transfected using Lipofectin (In vitrogen) into the SKOV-3 neomycin-resistant clonal derivative cell line, SKNV3.3. OBCAM sense and antisense-transfected clonal cell lines were then established.

2.7.2 Lipofection of plasmid DNA into cell lines

PvuI-linearised sense and antisense OBCAM constructs were transfected using Lipofectin (In vitrogen) into the SKOV-3 neomycin-resistant clonal derivative cell line, SKNV3.3. This technique utilises Lipofectin (BRL) which is a 1:1 (w/w) liposome formulation of the cationic lipids DOTMA and DOPE in water. $2x10^5$ cells per 60mm petri dish were seeded and incubated for 24-48 hours until 30-50% confluent. Lipofectin and plasmid were allowed to warm up to room temperature for at least 20 minutes prior to use. A similar length of incubation time for RPMI (serum free and 10%FCS, both antibiotic free) at 37^0C was allowed before use.100μl serum free RPMI was aliquoted into sterile microfuge tubes, two per lipofection. 2μg plasmid DNA was added to the first tube.10μl of Lipofectin (check) was put into the second tube. The solutions were gently mixed in a 5ml tube and incubated at room temperature undisturbed for 15 minutes. Immediately prior to lipofection, the recipient cultures were washed once in

serum free RPMI. 1.8 ml serum free RPMI was added to the plasmid / lipid suspension and the 5ml tube inverted gently several times. The resultant 2ml suspension was added to the recipient Petri dish and incubated at 37^0C for 6 hours. The suspension was then removed and 4ml of RPMI containing 10%FCS was added and the cells incubated without selective pressure for at least 48 hours to expand cell numbers. The cells were subcultured 1:5 into geneticin and zeocin selective media at previously optimised concentrations. Clones were picked by trypsinisation using fine tipped pastettes at 2-4 weeks. OBCAM sense and antisense transfected clonal cell lines were then established.

2.7.3 Zeocin Kill curves

Zeocin kill curves were performed to establish the optimal concentration needed for transfection selection. The chosen concentration was the lowest concentration that began to give massive cell death at about five days and killed all the cells witin two weeks. 1 x 10^3 SKNV3.3 cells were seeded per well into a 24 well plate. Varying concentrations of zeocin ranging from 0 – 500μg/ml were placed in each of 3 wells. The plates were refed and visualised uunder the microscope every three days for two weeks. The optimal antibiotic concentration of 125μg/ml was clear from inspection and therefore formal cell counts were not undertaken. Optimal geneticin concentrations had been previously determined in the lab prior to the creation of the SKNV3.3 cell line.

2.7.4 DNA miniprep

DNA was prepared from the transfected cell lines for use in PCR to identify successful integration of the transfected plasmid DNA.

Cells from a confluent 24 well plate were harvested and pelleted in a 1.5 ml microcentrifuge tube and frozen at -70^0C until adequate numbers of samples were accumulated. To make DNA, the Qiagen QIAamp DNA minikit was used. The cell pellet was resuspended in PBS to a final volume of 200µl and 20µl of proteinase K added. 200µl buffer AL was added and mixed by pulse vortexing for 15 seconds. Following a 10 minute incubation at 56^0C the tube was briefly centrifuged. 200µl of 100% ethanol was added and mixed by pulse vortexing for 15 seconds. After a brief centrifuge, the mixture was carefully applied to a QIAamp spin column in a 2ml collection tube. This was centrifuged for 1 minute at 8000rpm. The spin column was transferred to a fresh tube and the tube containing the filtrate discarded. 500µl of buffer AW1 was added and centrifuged for a further minute at 8000rpm. The filtrate was again discarded. 500µl buffer AW2 was added and centrifuged at 130000rpm for 3 minutes. The column was placed in an empty collection tube and centrifuged at 13000rpm for 1 minute. Finally, the column was placed in a collection tube and 50µl of buffer AE added and incubated for 5 minutes at room temperature. To elute the DNA, the tube was spun at 800rpm for 1 minute. This elution step was repeated to maximise the DNA yield.

2.7.5 Genomic PCR using zeocin primers

Genomic PCR using zeocin primers was performed on the DNAs made from the transfected cell lines. 2µl of DNA was used per 25µl PCR reaction. A Mg concentration of 1.5mM was used and a TD67-55 program run to amplify the 252bp product.

Table 2.12 Primers for detection of zeocin plasmid incorporation

Primer	Sequence 5'-3'	Acc. No	Nucleotide	Size bp
ZEO FOR	CAAGTTGACCAGTGCCGTTC	pcDNA3.1	2189-2208	252
ZEO REV	GAAGTTCGTGGACACGACCT	pcDNA3.1	2422-2441	

2.7.6 RNA preparation from the transfected cell lines

RNA from the transfected cell lines was prepared using the Stratagene Absolutely RNA RT- PCR miniprep kit. When the transfected cells were 70% confluent in a $25cm^3$ flask the media was poured off and a mixture of 4.2µl Beta mercaptoethanol and 600µl of lysis buffer added to cover the bottom of the flask. The cell lysate was mixed and collected by repeated pipetting and transferred to a 1.5ml microfuge tube. The tube was vortexed to homogenise the lysate. The lysates were frozen at -70^0C until a batch of lysates were ready to process. The homogenate was transferred to a prefilter spin cup seated in a 2ml receptacle tube. The tube was spun in a microcentrifuge at maximum speed for 5 minutes and the filtrate retained. An equal volume of 70% ethanol was added to the filtrate and the tube vortexed for 5 seconds. This mixture was transferred to a filter-matrix spin cup seated in a fresh 2ml receptacle tube. This was spun at maximum speed for 60 seconds. The spin cup was retained and the filtrate discarded. The spin cup was replaced in the receptacle tube. To DNase treat the sample, the following procedure

was followed. 600µl of 1x low salt wash buffer was added and spun at maximum speed for 60 seconds. Again the spin cup was retained and the filtrate discarded. The spin cup was replaced in the receptacle tube and spun at maximum speed for 2 minutes. The DNase solution was prepared by gently mixing 50µl DNase digestion buffer with 5ml of reconstituted RNAse- free Dnase1. This solution was added directly onto the fiber matrix inside the spin cup. The sample was incubated at 37^0C for 15 minutes in an air incubator. 600µl of 1x high salt buffer was added to the spin cup and spun at maximum speed for 2 minutes to dry the filter matrix. The spin cup was transferred to a clean 1.5ml microfuge tube and 60µl elution buffer added directly onto the center of the matrix of the spin cup. The tube was incubated at room temperature for 2 minutes and then spun at maximum speed for 1 minute.

The purified RNA in the elution buffer was quantified by measuring the optical density at 260 and 280nm.

$$\text{Concentration} = \frac{\text{OD260 x dilution factor x 40}}{1000} = \mu g/\mu l \text{ RNA}$$

Aliquots of RNA were stored at – 70C.

2.7.7 Determination of construct expression in OBCAM transfected cell lines

Semi-quantitative RT-PCR was used to determine expression of the sense and antisense constructs in the transfected cell lines. The OPCML F7/BGH primer pair was used to detect expression of the sense construct and OPCMLR3/BGH used to detect expression of the antisense construct.

Table 2.13 Primers for detection of construct expression

Primer	Sequence 5'-3'	Acc. No	Nucleotide	Size bp
OPCML FOR7	CCACTGGTCTGGATGGAATG	NM_002545.1	841 – 860	~320
BGH REV	TAGAAGGCACAGTCGAGG			
OPCML R3	CGTCACGTTGTCCATAGCTT	NM_002545.1	188-169	~230
BGH REV	TAGAAGGCACAGTCGAGG			

2.8 OBCAM Functional Analyses

2.8.1 Maintenance of cell lines

Cell lines were maintained in RPMI 1640 with heat inactivated 10% Fetal Calf Serum (FCS) and penicillin (100units/ml) streptomycin (100µg/ml) and G418 (3ml 20mg/ml per 100ml media = 600µg/ml) and Zeocin (In vitrogen) (125µl per 100ml media = 125µg/ml) as appropriate.

2.8.2 Growth Assay

Log phase cultures of parent and OBCAM transfected cell lines were harvested and $1x10^4$ cells in 1ml media seeded in triplicate into 24 well trays. Every third day, one plate of cells was harvested and counted. To harvest, the media was removed and the cells washed twice with PBS. 250µl trypsin was added and the cells incubated at 37^0C until completely detached. To resuspend the cells, 750µl of serum containing media was added to each well. The cells in each well were syringed 3 times through a 21gauge needle to create a single cell suspension. The media was replaced on the remaining plates every three days. The assay was performed three times.

2.8.2.1 Use of the Coulter counter

The Coulter counter was drained of Coulter Klenze and filled with saline. A background count was taken to ensure that the counter was working adequately. (High background counts suggest caution). To count, 200µl of trypsinised single cell suspension from each well was added to 9.8ml saline in a counting pot. The pot was capped and gently inverted twice to mix. After counting, the counter was flushed, drained and refilled with Coulter Klenze.

2.8.2.2 Analysis

The actual cell count was determined by multiplication of the count by the dilution factor, 5000. Linear plots were drawn allowing comparisons between parent/control cell lines and cell lines transfected with both sense and antisense OBCAM gene constructs.

2.8.3 Aggregation Assay

Log phase SKNV3.3 control and OBCAM transfected cells were trypsinised and resuspended in media containing 10% FCS. 1 x 10^6 cells were resuspended in 1ml of media and passed through a 21gauge needle to create a single cell suspension. Cell suspensions were then incubated in 5% CO_2 at 37 ^{0}C. At defined time points, aliquots were removed by a wide bore pipette, and single cells were counted with a haemocytometer.

2.8.4 Intraperitoneal and Subcutaneous Tumorigenicity Assays

Log phase SKNV3.3 control and OBCAM transfected cells were harvested and 5 x 10^6 cells injected either intraperitoneally (i.p.) or subcutaneously (s.c.) into the flanks of *nude* mice, with 3 i.p. and 6 s.c. injections per cell line. Size measurements of s.c.tumours were taken weekly for 4 weeks. Mice that received cells via i.p. injection were sacrificed at 9 weeks post injection and tumours removed from the peritoneal cavity, weighed and photographed.

2.8.4.1 Harvesting cells for xenograft experiments

Each cell line was bulked up in 175cm^3 flasks and cells were harvested in log phase at 70-80% confluence. Cells were washed twice with PBS and 2.5ml trypsin added to each flask. The flasks were incubated at 37^{0}C until the cells had detached. The cells were resuspended in RPMI containing 10% FCS and the cells from each cell line amalgamated. The cells were syringed four times through a 21gauge needle to create a single cell suspension and counted using a haemocytometer. The volume of cell suspension required was calculated and aliquoted into sterile universal containers and spun for 5 minutes at 2000rpm at room temperature. The supernatant was discarded and the cells resuspended by flicking. The cells were amalgamated into one universal per cell line by adding 10ml of serum free RPMI into the first universal and transferring it to the next until all the cells were in one universal. This process was repeated with another 10ml serum free RPMI. The cells were then spun twice more in serum free media to remove any remaining traces of fetal calf serum. The resulting cell pellet was resuspended in serum free media to give 5 x 10^6 cells per 100µl injection volume. This

suspension was put onto ice and taken immediately to the Biomedical Research Facility for injection into the nude mice by Alison Ritchie and Morwena Muir, Cancer Research UK, BRF.

2.9 OBCAM Demethylation in SKOV-3 with 5'-Aza 2'-deoxycytidine Exposure

$1x10^5$ SKNV3.3 cells (a neomycin-resistant, clonal SKOV-3 derivative) were seeded into a $25cm^3$ tissue culture flask in 10ml media. The media was replaced after 24 hours and 5'-aza 2'-deoxycytidine (Sigma) added to a final concentration of 20μM. A duplicate flask of control cells was not exposed to 5'-aza 2'-deoxycytidine. After 4 days, cells were harvested, DNaseI-treated total RNA prepared and first strand cDNA synthesised. β-Actin RT-PCR was performed to confirm the integrity of the 1st strand cDNA from control and 5'-aza 2'-deoxycytidine-treated SKVN3.3 cells.

OBCAM RT-PCR was performed with primers to amplify a 474bp product. Products from this reaction were size separated on a 2% agarose gel, Southern blotted overnight onto MSI nylon membrane and then UV crosslinked to the membrane. The blot was hybridised with a 395 bp OBCAM probe amplified by PCR from RNA from normal human ovary. The probe was purified through a QIAquick PCR purification column (Qiagen), and labelled with α^{32}P-dCTP. Following overnight hybridisation, the blot was washed to remove unbound probe, and the blot exposed to XAR-5 autoradiography film for 1 hour at –70°C and then developed.

Table 2.14 Primers for RT-PCR for use in demethylation experiment

Primer	Sequence 5'-3'	Acc.No	Nucleotide	Size bp
For	TACCATAGATGACCGGGTAA	NM_002545.2	221-240	474
Rev	TTCCGCACATCGGGCGCAGC	NM_002545.2	694-675	

Table 2.15 Primers for RT-PCR of OBCAM probe

Primer	Sequence 5'-3'	Acc.No	Nucleotide	Size bp
For	ATAGACCCTCGTGTGATCA	NM_002545.2	300-319	395
Rev	TTCCGCACATCGGGCGCAGC	NM_002545.2	694-675	

2.10 Reagents and solutions – manufacturers and suppliers

Tissue culture

5aza2deoxycytidine 50mg Sigma Cat No. A3656

Fetal calf serum PAA Laboratories

Geneticin GibcoBRL Life Technologies Cat No. 10131

Hygromycin B 1g Roche Cat No. 843555

Lipofectin Gibco BRL Life technologies Cat No 1892-011

Penicillin/streptomycin In vitrogen Cat No. 15140-122

PBS (Phosphate buffered saline) Oxoid 100tabs BR14a

RPMI 1640 Media with glutamine In vitrogen

Tissue culture plastics Falcon

Trypsin-EDTA 1x In vitrogen

Zeocin In vitrogen Cat No R250-05

ABI 310

Genescan 350 TAMRA PE Applied biosystems Cat No. 401736

310 capillaries 47cm x 50µM PE Applied biosystems Cat No. 402839

310 genetic analyser buffer 10x with EDTA PE Applied biosystems

PCR/RTPCR/sequencing

96 well plates, 0.2ml strips, 0.5ml microfuge tubes, 1.5ml eppendorfs AB Gene

10x buffer Roche

10x reaction bufferII PE Applied biosystems

100bp DNA ladder GibcoBRL Life Technologies

Absolutely RNA RT-PCR Miniprep kit Stratagene Cat No 400800

Agarose Sigma Cat No. A-9539

AmpliTaq Gold PE Applied biosystems

Big Dye PE Applied Biosystems Cat No.4303152

Chloroform Sigma C-2434

CpGenome DNA Modification kit Intergen

Diethlypyrocarbonate Sigma D-5758

Dynex plate sealers 1541892 Thermo Labsystems

dRhodamine Terminator Cycle Sequencing Ready Reaction Kit PE Applied Biosystems

Dynex plate sealers 1541892 Thermo Labsystems

Ethidium bromide Sigma Cat No. E1510

Filter tips Anachem

Magnesium 25mM PE Applied biosystems

Microseal "A" film GRI MJ Research Cat No. MSA-5001

Pellet paint Novagen 70748-3

PicTaq CRUK

QIAquick PCR purification kit QIAGEN Cat No. 28104

Sterile water Sigma Cat No. W4502

TRI Reagent Sigma T-9424

Ultrapure dNTP Pharmacia Biotech Cat No. 27-2035-02

SSCPE

Acrylogel3 BDH Laboratory supplies

10xTBE buffer

10 % AMPS Ammonium persulfate Sigma A-6738

TEMED (N,N,N',N'-Tetramethylethylenediamine) Sigma T-8133

10% ethanol

1% nitric acid

0.1% silver nitrate BDH Laboratory supplies

Developer

Fixer

Solutions

0.5M EDTA pH 8.0

186.1g disodium EDTA (Sigma E-5134)

-add ~ 800ml dH_2O

-add 20g NaOH pellets and mix until dissolved, using heat if necessary.

-adjust pH to 8.0 with NaOH pellets (Fisons)

Make up to 1l with dH_2O

10x TBE buffer

108 g Trizma base (Sigma E-1503)

55g boric acid (Sigma B-7901)

-add ~ 800ml dH_2O and stir until dissolved

-add 40ml 0.5M EDTA pH 8.0

Make up to 1l with dH_2O

10% AMPS

0.1g AMPS in 1ml dH_2O. Make up fresh for use each day.

SSCPE Loading buffer

19ml formamide

0.8ml 0.5M EDTA pH 8.0

50mg Bromophenol Blue (Sigma B-5525)

50mg Xylene Cyanol FF (Sigma X-0377)

SSCPE Staining Solutions

10% ethanol	250 ml
1% nitric acid	250 ml
0.1% silver nitrate (0.25g silver nitrate/250 ml dH_2O)	250 ml

Developer (15g anhydrous Sodium carbonate, 500ml dH_2O, 250 µl formaldehyde)500 ml

Fixer (100 ml methanol, 25 ml acetic acid, 7.5 ml glycerol + dH_2O to 250 ml)250 ml

20x TAE running buffer

145.2g Trizma base (Sigma E-1503)

34.26ml glacial acetic acid

60ml 0.5M EDTA pH8

Make up to 1.5litres with dH_20

PBS (Phosphate buffered saline)

10 tabs per litre dH_20

Autoclave 115^0C for 10 mins

0.1% DEPC H_20

100µl DEPC per litre dH_20

Leave to stand in glassware over night

Autoclave 120^0C for 1 hour 40 mins

3. RESULTS

LOH ANALYSIS

3 LOH ANALYSIS

3.1 LOH on chromosome 11q24-5 in epithelial ovarian cancer

Amplification of 16 polymorphic microsatellite markers from a panel of 112 normal/tumour DNA pairs was undertaken and LOH analysis performed using the Genescan software. Table 3.1 shows the results. A hatched box indicates that the patient was homozygous at that marker. A black box indicates loss of heterozygosity whilst an open box indicates retention of heterozygosity in the tumour at that marker. Not all markers were amplified from every DNA pair. Only a limited quantity of DNA from Aberdeen was available and therefore only a subset of markers was amplified from those DNAs.

3.2 Clinicopathological characteristics of study cohort

Table 3.2 shows the clinicopathological characteristics of study cohort.

3.3 Ordering of microsatellite markers on 11q24-5

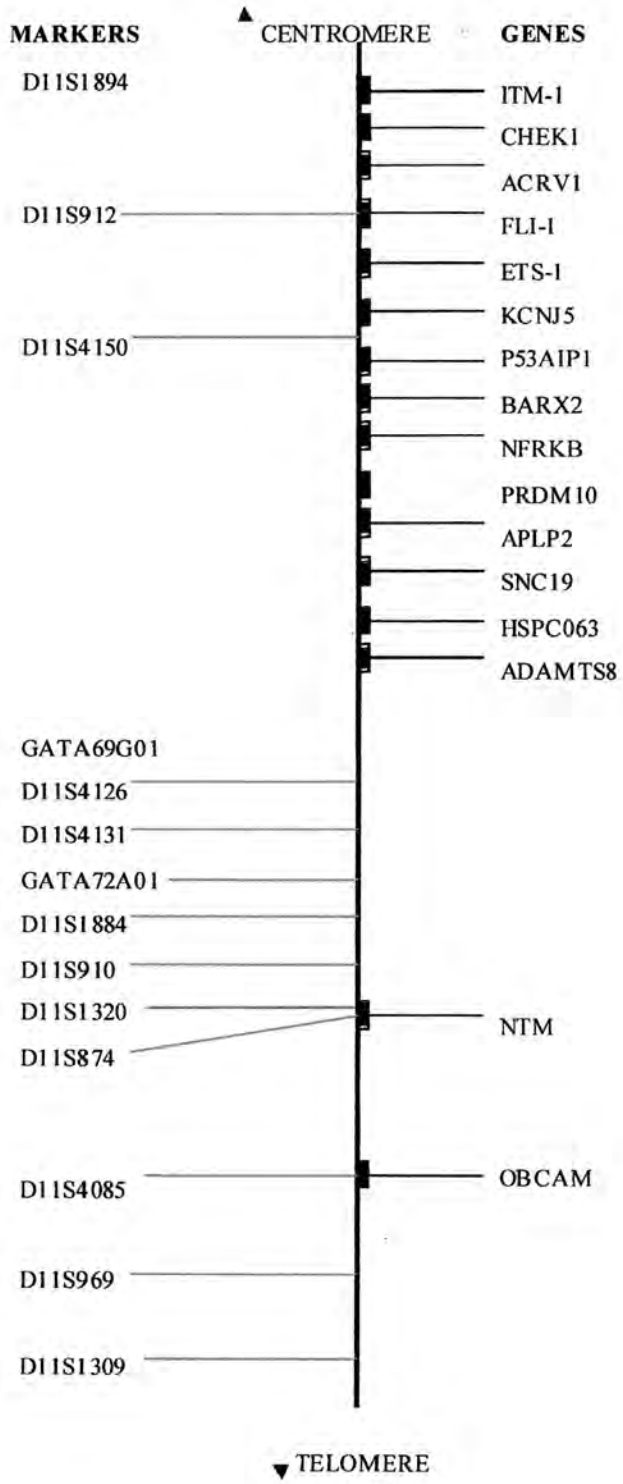
Figure 3.1 shows the predicted order of the microsatellite markers within the 11q24-5 region. This figure is not to scale. Throughout this research this map was constantly updated and rearranged as new data became available through the Human Genome Sequencing Project. This final marker order was the best prediction available based on data available at the middle of June 2002. The majority of markers were annotated on contig NT_009115.8. At this time this contig was 7715220bp long and was still unfinished, working draft sequence. Several of the markers used in this study were not yet annotated and the position of these markers was determined by blasting the

microsatellite sequences against the contig. Using this approach the position of GATA69G01 was unambiguously determined. Blast search of D11S4131 and GATA72A01 revealed several hits on the contig. This may be due to sequence duplication or alternatively be due to error in the working draft sequence. Combining this data and information obtained from previous physical mapping, these markers were placed in their most likely location on the map. Two markers, D11S4198 and D11S1895 were unable to be placed in this region of chromosome 11. This may be because these markers are actually out with the region. Alternatively, they may reside in locations that are not yet sequenced. These two markers will not be discussed further.

Table 3.2 Clinicopathological characteristics of study cohort

No. patients	112
Histology	
Serous	50
Endometrioid	27
Mucinous	14
Clear cell	12
Mixed endo /serous	3
Mixed endo/clear	2
Unclassified epithelial	2
Mixed serous / mucinous	1
Undifferentiated	1
Differentiation	
Well	11
Moderate	28
Poorly	65
Unknown	8
Stage	
I	35
II	13
III	55
IV	8
Unknown	1
Surgical Treatment	
Complete debulk	60
Partial debulk	9
No debulk	13
Unknown	30

Figure 3.1 Order of microsatellite markers and genes on chromosome 11q24-5



3.4 LOH rates at individual polymorphic markers

Table 3.3 shows the number of pairs analysed for each marker. It also documents the number of informative cases and the number of those cases that sustained LOH. If the background (random) rate of LOH is taken as the lowest detected rate of LOH, it can be seen that several markers from this region display modestly elevated rates of LOH. A striking feature of this data is the markedly elevated frequency of LOH at the marker D11S4085. Just over half of the patients who were informative at this marker had LOH in their tumours.

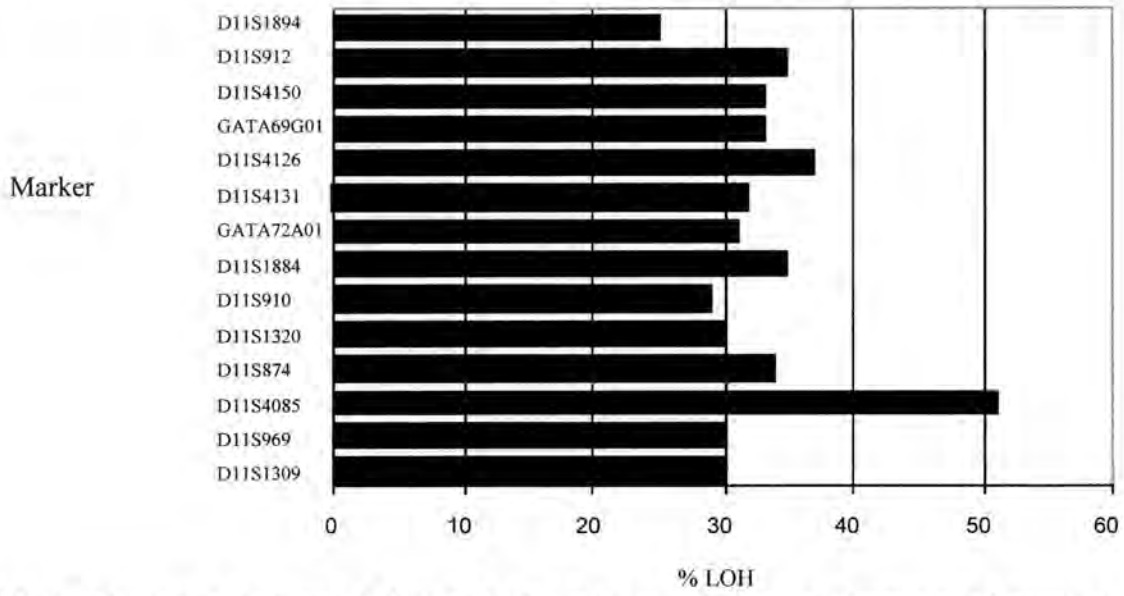
Table 3.3 LOH rates at individual polymorphic markers

	No.analysed	No.informative	No.LOH	%LOH
D11S1894	58	44	11	25
D11S912	80	68	24	35
D11S4150	74	66	22	33
GATA69G01	64	24	8	33
D11S4126	63	30	11	37
D11S4131	99	79	25	32
GATA72A01	94	39	12	31
D11S1884	54	26	9	35
D11S910	91	56	16	29
D11S1320	91	60	18	30
D11S874	100	53	18	34
D11S4085	97	68	35	51
D11S969	89	60	18	30
D11S1309	53	27	8	30
D11S1895	65	36	10	28
D11S4198	61	48	13	21

3.5 Graphical representation of the % LOH at each microsatellite marker

Figure 3.2 is a graphical representation of the % LOH at each microsatellite marker.

Figure 3.2 Percentage LOH at individual polymorphic markers



3.6 Relationship between LOH and the clinicopathological features of ovarian cancer

Investigation of the association of LOH at individual markers with the clinical and pathological variables of the cohort of patients was undertaken using the STATA statistics package. Correlation of LOH of any of the markers with FIGO stage, grade, histological type and debulk status was performed. There were several significant correlations associated with LOH at individual markers and these are shown in table 3.4. Table 3.4 also includes correlations that showed a trend towards significance.

LOH at D11S4150 was significantly associated with non clear cell histological type ($p = 0.045$). There was a trend towards significance for LOH and non clear cell histology at the markers D11S4131 ($p = 0.051$), D11S1884 ($p = 0.063$) and D11S1894 ($p = 0.085$). The explanation for these correlations is uncertain. In general, tumours of clear cell histological type are regarded as a poor prognosis group of tumours. LOH at D11S4131

was significantly associated with non-serous histological type (p= 0.015). LOH at D11S4150 was significantly associated with mucinous histology (p = 0.045), with LOH at D11S912 showing a similar trend (p = 0.083). LOH at D11S969 was significantly associated with late stage (non stagel) disease (p = 0.0085).

Table 3.4 Statistical association between defined polymorphic loci and clinicopathological features of ovarian cancer

Prognostic parameter assoc'd LOH	Marker	No. Informative	Fisher's p-value
Non clear cell	D11S4131	79	0.051
Non clear cell	D11S4150	66	0.045
Non clear cell	D11S1884	26	0.063
Non clear cell	D11S1894	44	0.085
Non clear cell	D11S4126	30	0.014
Nonserous histology	D11S4131	79	0.015
Mucinous histology	D11S4150	66	0.045
Mucinous histology	D11S912	68	0.083
Late stage disease	D11S969	60	0.0085

3.7 Relationship between LOH and survival of patients with ovarian cancer

Kaplan-Meier survival analysis using the log rank test for statistical significance showed significant adverse survival with LOH at the markers D11S4150 (p = 0.0336), D11S4131 (p = 0.0170) and D11S1309 (p = 0.0231). The survival curves for these markers are shown below. Table 3.5 details the p value for each marker produced by the log rank test. This table details the number of informative cases at each marker along with the number of patients in each of the heterozygous and LOH groups that had died (Dead/HET and Dead/LOH). Survival data was not available for two patients in the

cohort and therefore the number of informative cases at each marker in this analysis varies by up to two from table 3.3. The Kaplan-Meier survival curves for LOH at D11S4150, D11S4131 and D11S1309 are shown in figures 3.3, 3.4 and 3.5.

Table 3.5 Statistical association between LOH of defined polymorphic loci and survival of patients with ovarian cancer

Marker	No. informative	Dead/Het	Dead/LOH	P value
D11S1894	44	17/33	6/11	0.5325
D11S912	68	19/44	11/24	0.6489
D11S4150	**66**	**16/44**	**13/22**	**0.0355**
GATA69G01	25	5/16	4/8	0.5411
D11S4126	30	11/19	6/11	07346
D11S4131	**77**	**25/53**	**17/24**	**0.0170**
GATA72A01	38	14/27	6/11	0.3408
D11S1884	26	9/17	7/9	0.2255
D11S910	54	22/39	6/15	0.5050
D11S1320	59	19/41	10/18	0.2907
D11S874	51	18/34	9/17	0.9928
D11S4085	66	18/33	14/33	0.5931
D11S969	59	21/41	11/18	0.0528
D11S1309	**27**	**10/19**	**6/8**	**0.0231**
D11S1895	36	11/26	6/10	0.0302
D11S4198	48	15/35	7/13	0.2953

Figure 3.3 Kaplan – Meier survival estimate – D11S4150

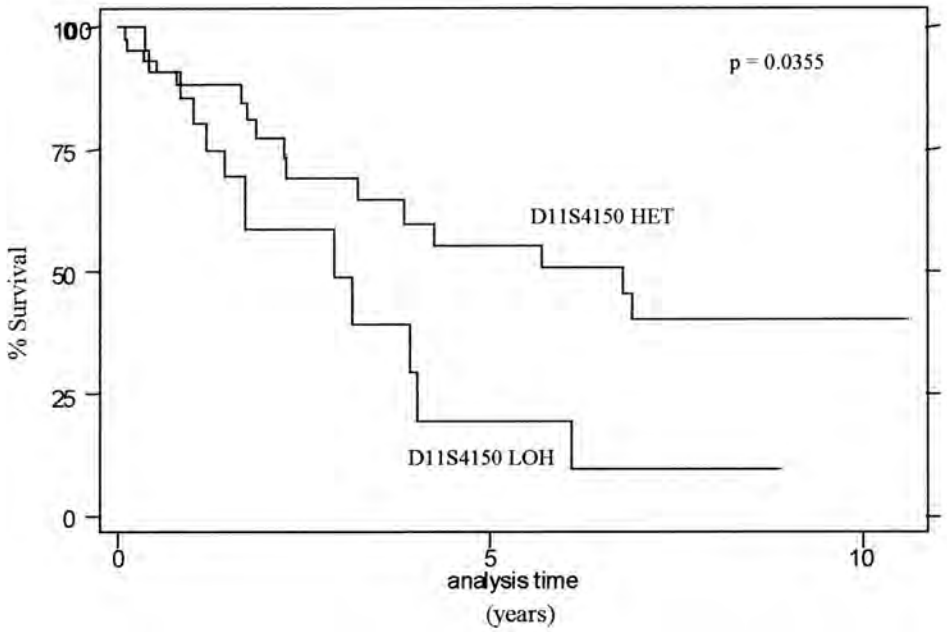


Figure 3.4 Kaplan – Meier survival estimate – D11S4131

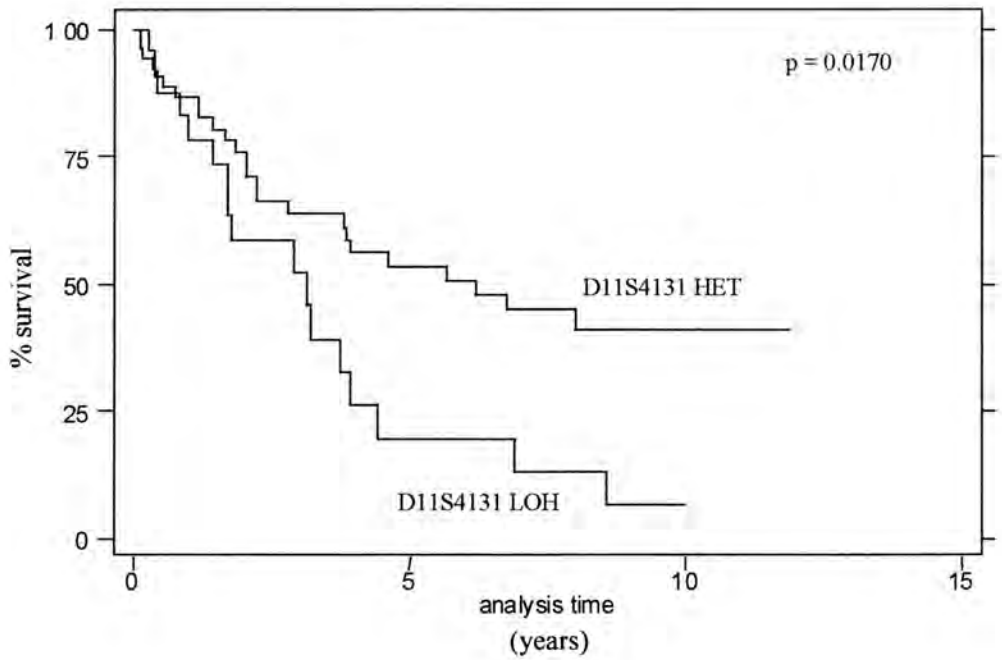
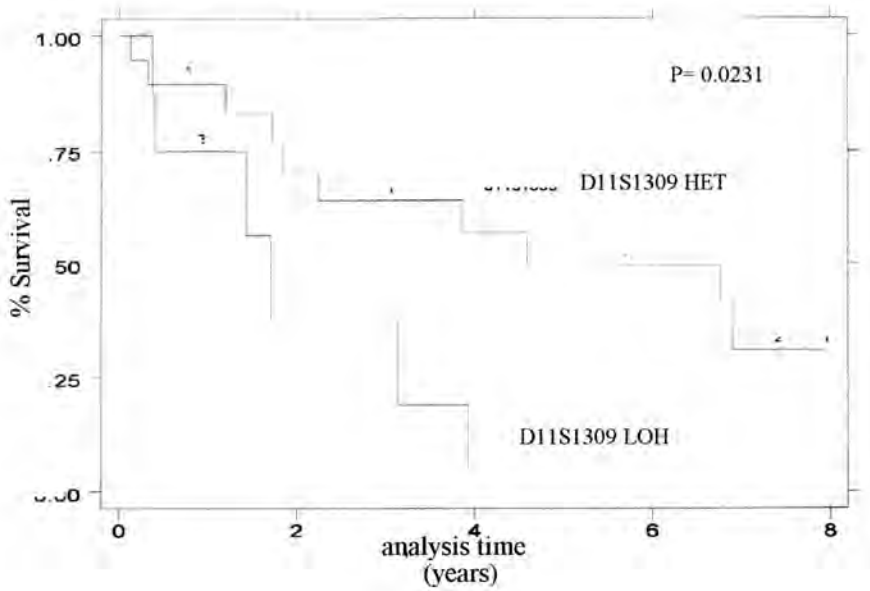


Figure 3.5 Kaplan – Meier survival estimate – D11S1309



3.8 Multivariate analysis of prognostic parameters in patients with epithelial ovarian cancer

Multivariate analysis was carried out on a subgroup of 65 patients with ovarian cancer. The analysis had to be restricted to this set of patients as there was missing data for some of the patients from Aberdeen. The results are shown in table 3.6. As would be expected stage and debulk status were independent prognostic markers. Of great interest is that LOH of D11S4131 appears to be an independent prognostic indicator.

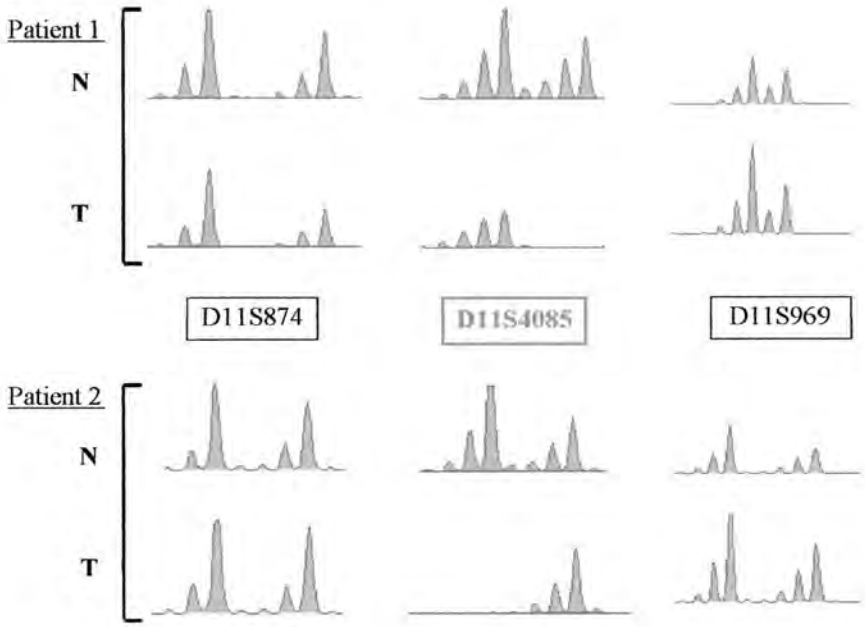
Table 3.6 Multivariate analysis – Statistical association between LOH of polymorphic loci and clinicopathological features of ovarian cancer

Prognostic factor	Hazards Ratio	95% CI	P value
Stage I + II	1.000		
StageIII + IV	4.419	(1.485, 13.146)	0.0076
Other histology	1.000		
Serous histology	2.502	(1.165, 5.373)	0.0190
D11S4131 HET	1.000		
D11S4131 LOH	3.53	(1.486, 8.383)	0.0043
Debulk complete	1.000		
Debulk partial/none	3.245	(1.193, 8.827)	0.0210

3.9 LOH at D11S4085

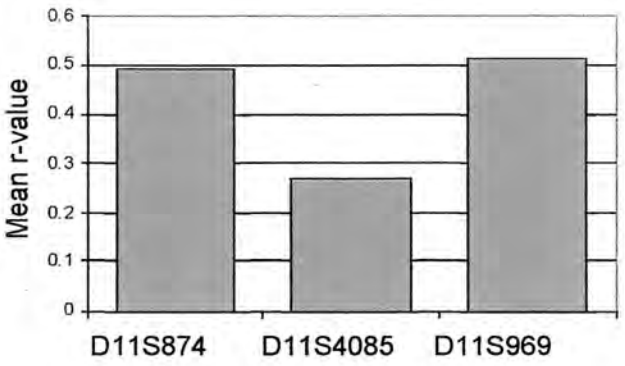
Figure 3.6 shows an example of the data that was analysed using the genescan software. An ascending group of peaks represents one allele. These patients are heterozygous at the three markers shown. In these patients' tumours, there is complete loss of one allele at D11S4085 with retention of heterozygosity at the flanking markers. LOH at D11S4085 was frequently complete, indicating a lack of tumour cell heterogeneity suggesting that this event occurs early in the development of the tumours.

Figure 3.6 Examples of LOH data generated by the Genescan software



As illustrated in figure 3.7, the mean level of allele imbalance (r) at D11S4085 was lower than that of the flanking markers.

Figure 3.7 Mean r-value at D11S4085 and the flanking markers D11S874 and D11S969



3.10 Identification of a consensus region of deletion involving D11S4085

Having identified a high rate of LOH at the marker D11S4085, a closer inspection of the LOH data (see table 3.1) revealed a consensus region of loss centred on the marker D11S4085. The shortest region of overlap was found to lie between the markers D11S874 and D11S969. This region is approximately 1Mb in size.

An estimate of the frequency of loss of this region in DNA from this cohort of patients with epithelial ovarian cancer was made. To determine the LOH rate of this region, as well as including cases informative at D11S4085, homozygous cases at this marker were included in the analysis if they had clear loss or retention of heterozygosity of both the flanking markers as this was felt to be indicative of the likely status at D11S4085. 42/79 informative cases (53%) had LOH of this region.

3.11 Relationship between LOH at Region 4085 and D11S4085 and survival of patients with ovarian cancer

As can be seen in figure 3.8, there was no association between LOH of this region and adverse survival. Figure 3.9 shows the Kaplan-Meier curve for the individual marker D11S4085 revealing that there is no association with adverse survival.

Figure 3.8 Kaplan – Meier survival estimate – Region 4085

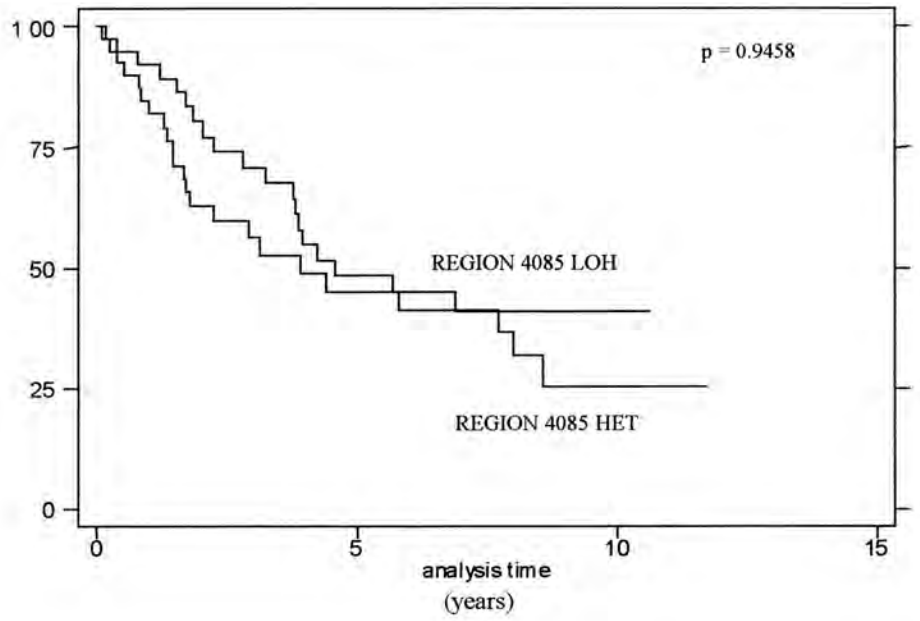


Figure 3.9 Kaplan – Meier survival estimate – D11S4085

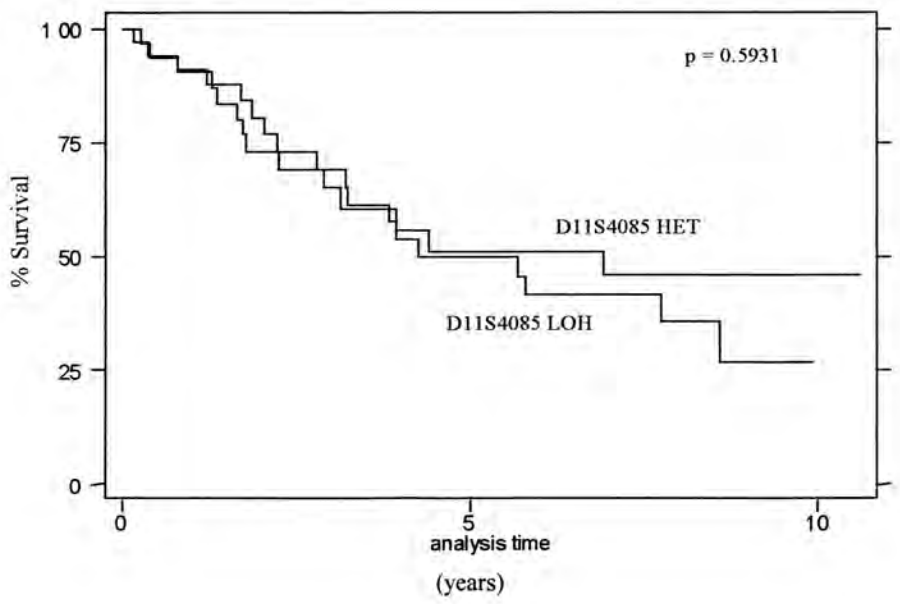


Table 3.7 documents the proportion of patients with loss and retention of heterozygosity who had died and the p value as determined by the log rank test.

Table 3.7 Statistical association between LOH at region 4085 and survival of patients with ovarian cancer

Marker	No. informative	Dead/Het	Dead/LOH	P value
Region 4085	78	22/38	20/40	0.9458

4. RESULTS

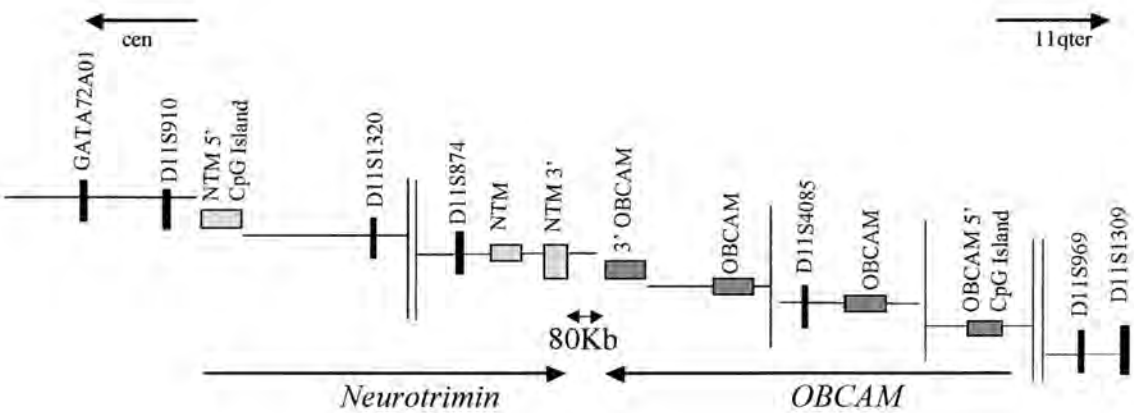
OBCAM – A CANDIDATE TUMOUR SUPPRESSOR GENE

4 OBCAM – A CANDIDATE TUMOUR SUPPRESSOR GENE

4.1 Identification of a gene in Region 4085

Identification of a high frequency of almost complete LOH at D11S4085 suggested that loss of a gene near this marker was likely to be an early event in the development of ovarian carcinomas. Using bioinformatics analysis, D11S4085 was found to lie within the second intron of a gene called Opioid Binding cell Adhesion Molecule (OBCAM) (see figure 3.1). Further analysis revealed that OBCAM lies telomeric to a gene called Neurotrimin (NTM). NTM and OBCAM are both members of the IgLON family of cell adhesion molecules. Their extensive homology and close proximity suggests that these genes arose as a result of a gene duplication event. Figure 4.1 shows the orientation of these two genes on chromosome 11q24 and indicates the positions of the associated polymorphic microsatellite markers used in the LOH analysis.

Figure 4.1 Localisation and orientation of OBCAM and NTM on chromosome 11q24-5



D11S1320 and D11S874 lie within the NTM gene. Calculation of the LOH rate of NTM is rather more difficult than defining the rate of OBCAM LOH because the NTM gene spans two polymorphic markers. The most conservative estimate of LOH at NTM is determined by identifying all cases that are informative at either or both markers. The LOH rate is calculated as the number of cases displaying LOH at either or both of these markers. Using this method, the LOH rate at NTM is 29/77 cases (38%). OBCAM LOH occurred frequently in the absence of NTM LOH (12/31 39%) whereas NTM LOH rarely occurred in the absence of OBCAM LOH (1/26 4% (p= 0.0001).

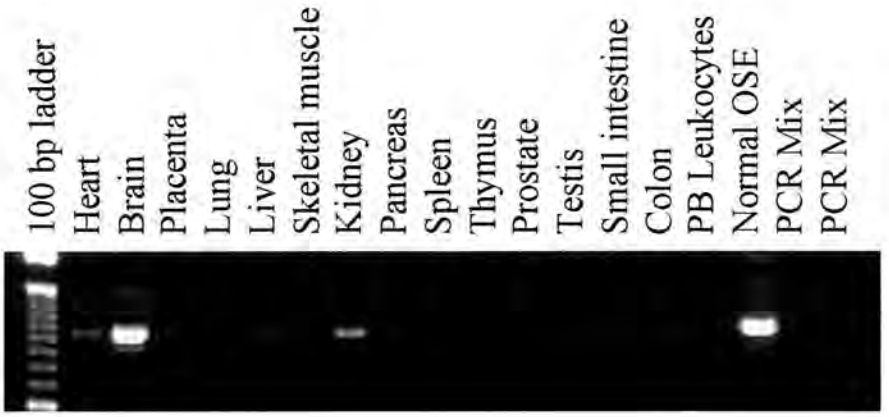
This suggests that LOH targets OBCAM rather than NTM in epithelial ovarian cancer and therefore OBCAM was taken forward for investigation as the candidate tumour suppressor gene from this region.

4.2 Expression of OBCAM in normal ovary, normal tissues, cell lines and tumour samples

4.2.1 Expression of OBCAM in normal tissues

The combination of OPCMLF1/OPCMLR1 primers amplifies the full length OBCAM mRNA transcript (1076bp). Figure 4.2 shows strong OBCAM expression in the normal ovarian surface epithelium and in the brain. Weaker expression of OBCAM was detected in heart, placenta, liver, kidney, testis and colon.

Figure 4.2 Semi-quantitative RT-PCR expression of OBCAM in normal tissues

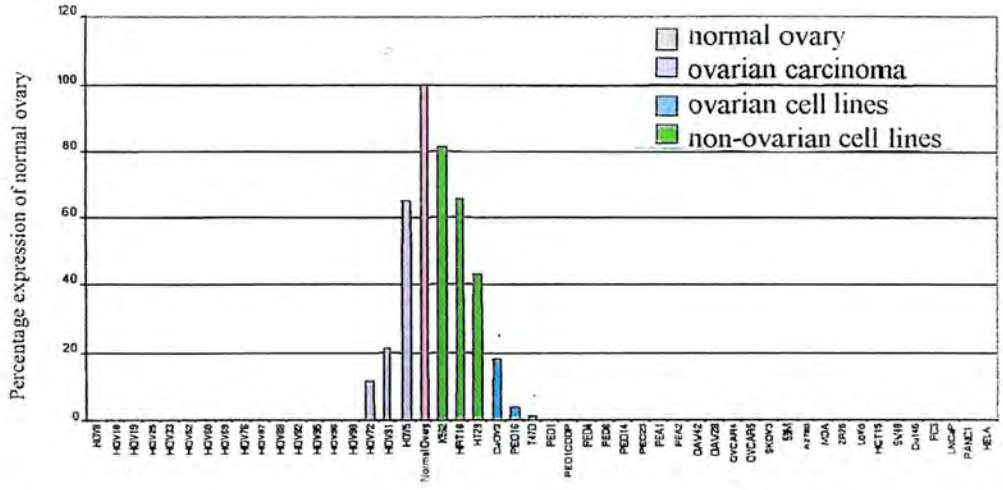


4.2.2 Expression of OBCAM in cell lines and primary ovarian tumours

Quantitative RT-PCR using a Light Cycler was performed on a series of 18 human primary ovarian tumours (HOVs), and 17 ovarian and 14 non-ovarian (breast, colorectal, prostate, pancreas, lung and myeloid) cancer cell lines. In this RT-PCR, a 224bp product was amplified using the OPCMLF4/OPCMLR4 primer pair. Figure 4.3 shows the result of this assay. OBCAM expression (normalised for Actin expression) was completely abolished in 16/18 (89%) primary ovarian tumours. No OBCAM expression was detectable in 15/17 (88%) of ovarian cancer cell lines. In the two expressing ovarian cancer cell lines, expression was 5% (PEO16) and 19% (CaOV3) of normal total ovary. In non-ovarian cancer cell lines, OBCAM expression was undetectable in 10/14 (71%) cell lines. Of the four non-ovarian cell lines with detectable OBCAM expression, three had levels that were not dissimilar to that of the control ovarian sample. The normal level of expression for these tissues (breast, colon and myeloid) however is unknown. In

essence, OBCAM expression is abrogated in the majority of primary ovarian tumours and ovarian cancer cell lines.

Figure 4.3 Quantitative RT-PCR expression of OBCAM in human ovarian tumours and ovarian and non-ovarian cell lines



4.3 Identification of the OBCAM CpG island and determination of its methylation status

4.3.1 Identification of the OBCAM CpG island

Having identified that expression of OBCAM is abrogated in the majority of ovarian cancer cell lines and ovarian tumours, it was necessary to investigate the nature of this silencing of expression. A proposed mechanism for this silencing was somatic methylation of the OBCAM CpG island. Using BLAST homology searches, the 5' nucleotide sequence of OBCAM mRNA, corresponding to a predicted first exon, identified a BAC clone (GenBank Accession AC027631) that is contained within the chromosome 11q25 Human Genome Contig Sequence NT_030800. Analysis of the

available sequence of this BAC clone with the GRAIL program CpG algorithm predicted a CpG island spanning 1kb approximately 500bp upstream of the translation start site of OBCAM.

Methylation Specific PCR (MSP) primers were designed to distinguish methylated (M) from unmethylated (U) alleles of the OBCAM CpG island sequence following sodium bisulphite modification of genomic DNA. An MSP assay that amplified a 134bp fragment was used to determine the methylation status of the OBCAM CpG island.

4.3.2 The OBCAM CpG island is unmethylated in normal human ovary

MSP of bisulphite-treated genomic DNAs extracted from normal ovaries from 5 women who underwent oophorectomy for non-malignant conditions showed that the OBCAM CpG island is normally unmethylated. Bisulphite sequencing across the 529bp OBCAM MS-PCR product from two of these normal ovaries showed sequence that was unmethylated at almost every one of the 58 CpGs within this region.

4.3.3 OBCAM is frequently somatically methylated in primary ovarian tumours

MSP was performed to detect methylated and unmethylated alleles of OBCAM in 69 matched normal/ovarian tumour DNA pairs. A representative set of MSP assays on 9 normal/tumour pairs including control universally methylated DNA and normal ovary DNAs is shown in Fig 4.4. The OBCAM CpG island is somatically methylated in 83% (57/69 cases) of ovarian primary tumours. In a subset of 26 samples for which both one stage MSP and LOH data was available, the MSP rate was 90% (9/10) where there was

LOH of OBCAM. The MSP rate was 69% (11/16) in those cases where there was no LOH of OBCAM.

Figure 4.4 Representative data from the OBCAM MSP assay

4.3.4 The OBCAM CpG Island is Methylated in Ovarian Cancer Cell Lines

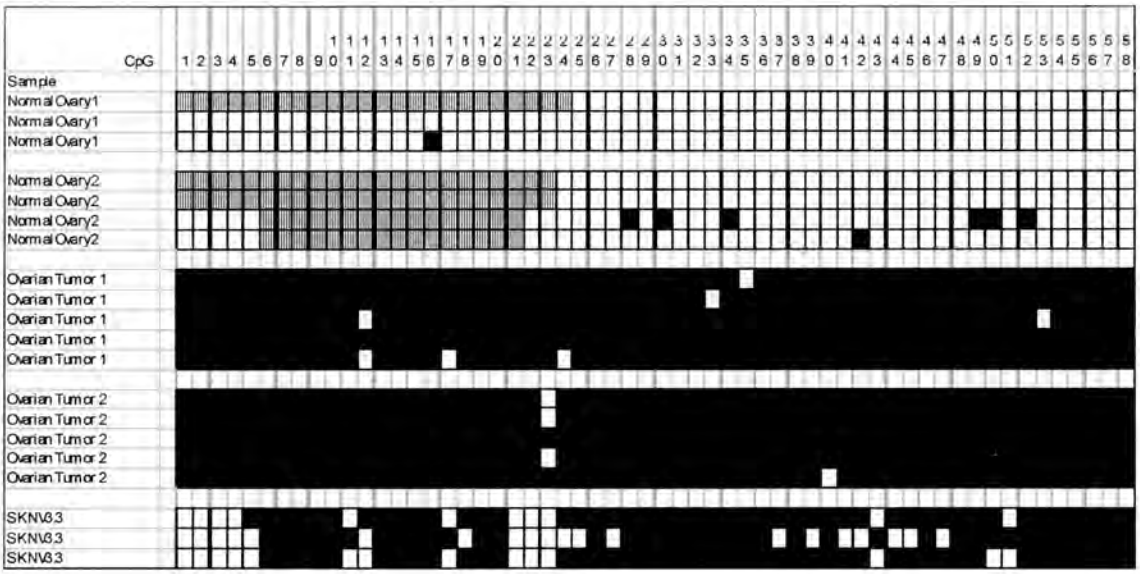
The OBCAM CpG island was found by MSP to be methylated in 82% (14/17) ovarian and in 82% (14/17) non-ovarian (breast, lung, colon and prostate) cell lines. The level of OBCAM expression in this cell line panel had previously been determined by quantitative RT-PCR (Fig 4.3) and therefore correlation between OBCAM methylation and expression was possible. Comparison of the MSP data with the quantitative RT-PCR expression profile revealed a significant correlation between OBCAM CpG island methylation and absence of detectable expression (P=0.02). Furthermore, five normal ovaries showed no evidence of CpG island methylation and expressed OBCAM.

4.3.5 Bisulphite sequencing of the OBCAM CpG island

From representative samples, an overlapping 529bp MSP product was amplified that contains 58 CpGs. This larger product was then sequenced to ascertain the extent of

methylation within the CpG island itself as opposed to just that of the primer binding sites. Bisulphite sequencing across the 529bp OBCAM MS-PCR product from two normal ovaries showed sequence that was unmethylated at almost every one of the 58 CpGs within this region. Bisulphite sequencing of the 529bp MSP product amplified from a sample of primary ovarian tumor DNAs demonstrated that the OBCAM CpG island was extensively methylated across 58 consecutive CpGs. This data is shown in figure 4.5. ■ denotes methylated C and □ denotes unmethylated C of a CpG dinucleotide. The shaded boxes represent CpGs for which the methylation status was undeterminable.

Figure 4.5 Bisulphite sequencing data from the OBCAM CpG island



4.3.6 Demethylation and Re-expression of OBCAM in SKNV3.3

In order to confirm that methylation was the mechanism underlying the repression of OBCAM expression, SKNV3.3 cells were exposed to the demethylating agent 5'-aza-2'-

deoxycytidine. The cell line SKNV3.3 is a neomycin resistant clonal derivative of the SKOV3 ovarian cancer cell line. SKNV3.3 does not express OBCAM as determined by quantitative RT-PCR. MSP and bisulphite sequencing have shown the OBCAM CpG island to be methylated at almost every CpG across the 529bp amplified fragment (see fig 3.14). Re-expression of OBCAM is clearly evident in the treated SKNV3.3 cells. In contrast, no OBCAM expression is detectable in control SKNV3.3 cells (Fig. 4.6). Actin RT-PCR confirmed the integrity of the isolated RNA and 1st strand cDNA. As SKVN3.3 cells do not express OBCAM, this cell line was selected for subsequent functional studies following transfection with OBCAM.

Figure 4.6 Re-expression of OBCAM following deoxyazacytidine exposure

4.4 Mutation screening of the OBCAM gene using SSCPE

4.4.1 Mis-sense mutations identified in the OBCAM gene

SSCPE was employed as a method for the identification of somatic mutations within the coding region of the OBCAM gene. SSCPE analysis for each OBCAM exon was performed on DNAs from 212 EOCs and 116 cancer cell lines. In keeping with the high rate of LOH and CpG island methylation observed for OBCAM, we found a low

frequency of somatic mutation. Surprisingly, no polymorphisms or somatic silent mutations were identified in this series. Analysis of the 212 tumour samples by SSCPE identified a single somatic mis-sense OBCAM mutation associated with a primary epithelial ovarian cancer. Two mis-sense mutations were identified from the panel of cell lines. The three mutations are documented in table 4.1.

Table 4.1 Mis-sense mutations identified in the OBCAM gene

Sample Name	Tumour Cell line	Exon	Nucleotide Position NM_002454.2	Change	Amino Acid	Functional Domain
PEO1	Ovary	2	334	C --> G	Pro --> Ser Codon 95	1st Ig domain
5637	Bladder	4	621	G --> T	Asp --> Tyr Codon 191	2nd Ig domain
KG1	AML	6	967	A --> G	Asn --> Ser Codon 306	Glycosylation site 3' to 3rd Ig domain

4.4.2 OBCAM Somatic Mutation in a Primary Ovarian Tumour

In the primary ovarian tumour, PEO1, a heterozygous peak corresponding to the presence of both a C and a G nucleotide at position 334 in codon 95 (NM_002545.2) indicates a transversion in the primary tumour (Fig4.7). This position is homozygous for C in the patient's normal DNA (marked by *). The mutation lies within the first Ig

domain of OBCAM. The original paper describing the cloning of OBCAM (Shark and Lee 1995) indicated that OBCAM consists of three "C2" like Ig domains. Analysis by Paul Bates, Cancer Research UK, Biomolecular Modelling Lab, indicates that the three domains are better classified as belonging to the "I" set of Ig domains. The "I" domain set is common for Ig domains within cell surface adhesion and receptor molecules (Harpaz and Chothia 1994). Based on this description of each domain P95 is predicted to lie within a beta turn between the D and E beta strands of the first Ig domain. The three-dimensional molecular model for this domain (Fig4.8) suggests that the P95R mutation would cause the hydrophilic arginine residue to point across the BED face of the domain (beta sheet consisting of beta strands B, E and D), and therefore, may interfere with any protein-protein interactions utilising this face.

Figure 4.7 Normal and tumour DNA sequence from the same patient reveals a somatic mis–sense mutation

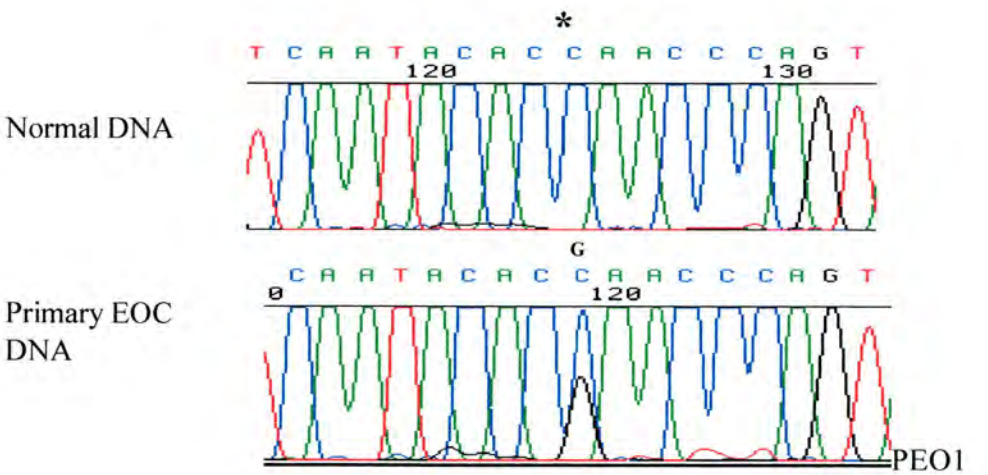
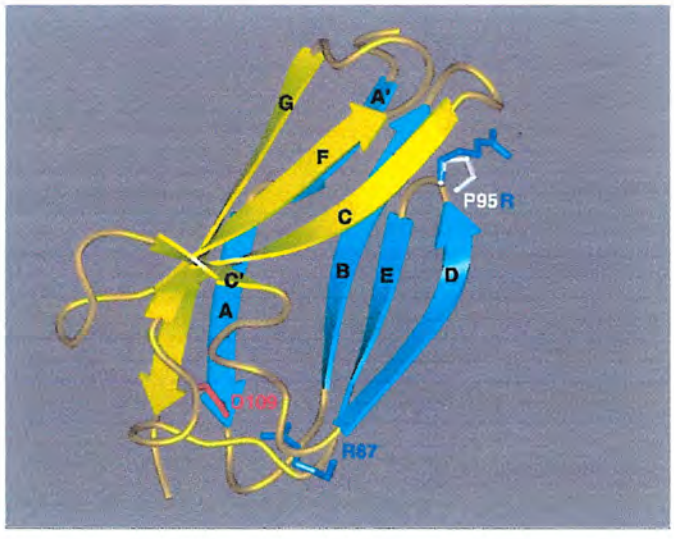


Figure 4.8 Biomolecular ribbon model of the first Ig domain of human OBCAM



4.4.3 SSCP identifies two mis-sense mutations in cancer cell lines

Two mis-sense mutations were identified in the panel of cancer cell lines. It is not known whether these represent somatic changes and modelling analysis has therefore not been undertaken. The first mutation in K5637, a cell line derived from a transitional cell carcinoma, shows a heterozygous peak corresponding to the presence of both G and a T nucleotide in exon 4 at nucleotide 621 in codon 191 (NM_002545.2). This codon is part of the second immunoglobulin domain of OBCAM and the mutation results in the substitution of a hydrophilic, charged aspartate residue with a hydrophilic, neutral tyrosine residue. The second mutation in KG1, a cell line derived from an acute myeloid leukaemia, also shows a heterozygous peak corresponding to the presence of both an A and a G nucleotide in exon 6 at nucleotide 967 in codon 306 (NM_002545.2). The affected amino acid is a predicted glycosylation site that lies carboxy terminal to the

third immunoglobulin domain of OBCAM. This mutation results in the substitution of a hydrophilic, neutral asparagine residue with a serine residue, another hydrophilic neutral residue.

Figure 4.9 5637 cell line DNA sequence reveals a mis-sense mutation in the OBCAM gene

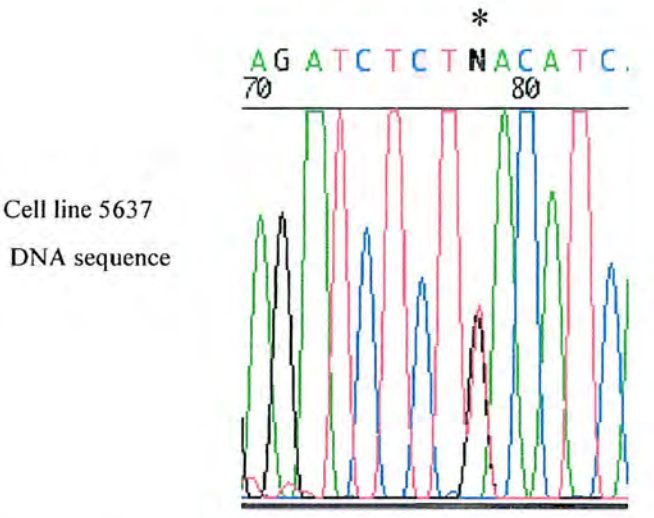
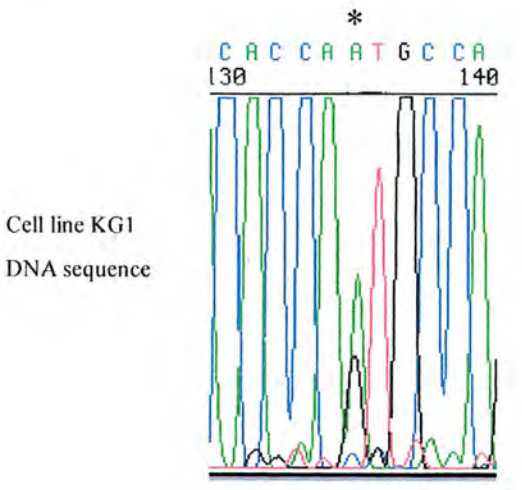


Figure 4.10 KG1 cell line DNA sequence reveals a mis-sense mutation in the OBCAM gene



4.5 Homology of OBCAM between species

The Clustalx1.8 multiple sequence alignment program was used to investigate the homology of OBCAM between species. OBCAM has been cloned in Rattus norvegicus (RN), Bos taurus (BT) and Gallus gallus (GG). Homologues have also been identified in Drosophila melanogaster (DM) and Caenorhabditis elegans (CE).

Inspection of the multiple sequence amino acid and nucleotide alignments of OBCAM in these different species immediately reveals the high homology of OBCAM between species (figs 4.11 and 4.12). This homology exists at both the amino acid and the nucleotide level.

The protein in mammals and GG is 345/344 amino acids long. OBCAM in DM is 315 amino acids long whilst a larger protein of 496 residues is seen in CE. The phylogenetic tree shown in figure 4.13 was drawn using the NJ tree program from within the Clustalx1.8 program. The length of branches is directly proportional to the divergence between species. The closest homologue to human OBCAM (HS) is that of RN. The LALIGN program was used to quantify the extent of homology in OBCAM between species. The results obtained using this program are shown in table 4.2. Analysis of homology was performed at both the amino acid and nucleotide sequence level. The amino acid or nucleotide overlap over which the homology was identified is included in the table. It can be seen that OBCAM in HS, RN and BT is highly conserved at the amino acid level. There is only marginally less conservation at the nucleotide level between these species. There is significantly less homology between HS and DM and CE.

Figure 4.13 OBCAM phylogenetic tree

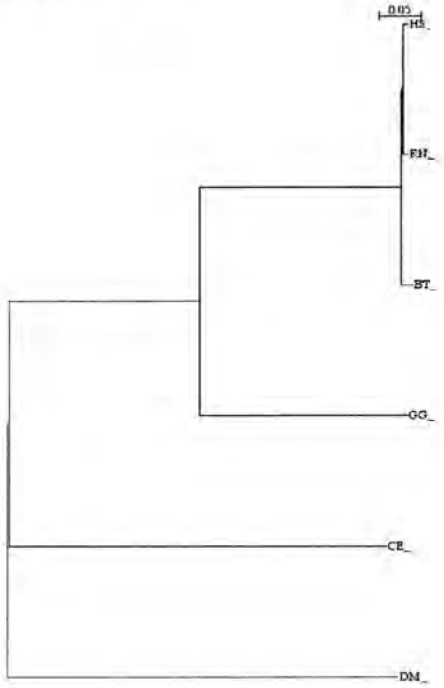


Table 4.2 Amino acid and nucleotide homology of OBCAM between species

	HS OBCAM amino acid sequence		HS OBCAM nucleotide sequence	
	% homology	Aminoacid overlap	% homology	Nucleotide overlap
RN	98.3	345	92.6	1038
BT	97.7	345	90.6	1038
GG	84.9	345	79.4	1038
DM	30.4	296	51.5	747
CE	25.9	317	51.8	583

In attempt to determine the significance of the presence of the three mis-sense mutations detected in the ovarian tumour and two cell lines, homology between these nucleotides and their associated amino acid codons was examined. Table 4.3 shows the presence or absence of homology at each position between species. Y = nucleotide or amino acid conserved at that position. N = nucleotide or amino acid not conserved at that position.

/ = gap in sequence. 1= mutation at codon 95, 2 = mutation at codon 191, 3 = mutation at codon 306. All three mutations are found at sites that are conserved between HS, RN and BT. The second and third mutations are also conserved in GG. This data suggests that these are functionally important nucleotides.

Table 4.3 Amino acid and nucleotide conservation of positions of identified mutations in the OBCAM gene

OBCAM	HS			HS		
	Aminoacid			Nucleotide sequence		
	1	2	3	1	2	3
RN	Y	Y	Y	Y	Y	Y
BT	Y	Y	Y	Y	Y	Y
GG	N	Y	Y	N	Y	Y
DM	N	N	N	Y	Y	Y
CE	/	N	N	Y	Y	N

4.6 Homology of OBCAM with other human IgLON family members

Inspection of the Clustalx amino acid and nucleotide alignments reveals high homology between OBCAM and the other two human IgLON family members, NTM and LAMP (figs 4.14 and 4.15, O= OBCAM, N=NTM, L=LAMP).

On inspection of the nucleotide sequence alignment, a striking feature is the 100% homology of the first 170 nucleotides of OBCAM and NTM. This high conservation between family members indicates that this region is functionally important.

The LALIGN program was used to quantify the extent of homology of OBCAM with the other two human IgLON family members. Table 4.4 documents the % homologies

identified using this program and the amino acid/nucleotide overlap over which the homology was identified.

Table 4.4 Amino acid and nucleotide homology between OBCAM and other human IgLONs

	HS OBCAM aminoacid sequence		HS OBCAM nucleotide sequence	
	% homology	Aminoacid overlap	% homology	Nucleotide overlap
NTM	76.5	345	76.4	1046
LAMP	56.2	322	65.3	1012

Table 4.5 shows the extent of conservation of the nucleotide sequence and amino acid residue at the position of the three mutations. Interestingly the position of the third mutation is conserved between IgLONs at the amino acid and nucleotide level. The codon determined by the third mutation is therefore conserved not only between species but also between IgLON family members. This suggests that disruption at this position is likely to be deleterious to protein function.

Table 4.5 Amino acid and nucleotide conservation of positions of identified mutations in OBCAM between IgLONs

HS	OBCAM			OBCAM		
	Aminoacid			Nucleotide sequence		
	1	2	3	1	2	3
NTM	N	N	Y	N	Y	Y
LAMP	N	N	Y	Y	Y	Y

4.7 Functional analysis of OBCAM in SKOV3 ovarian cancer cells

4.7.1 OBCAM Transfection into SKOV3

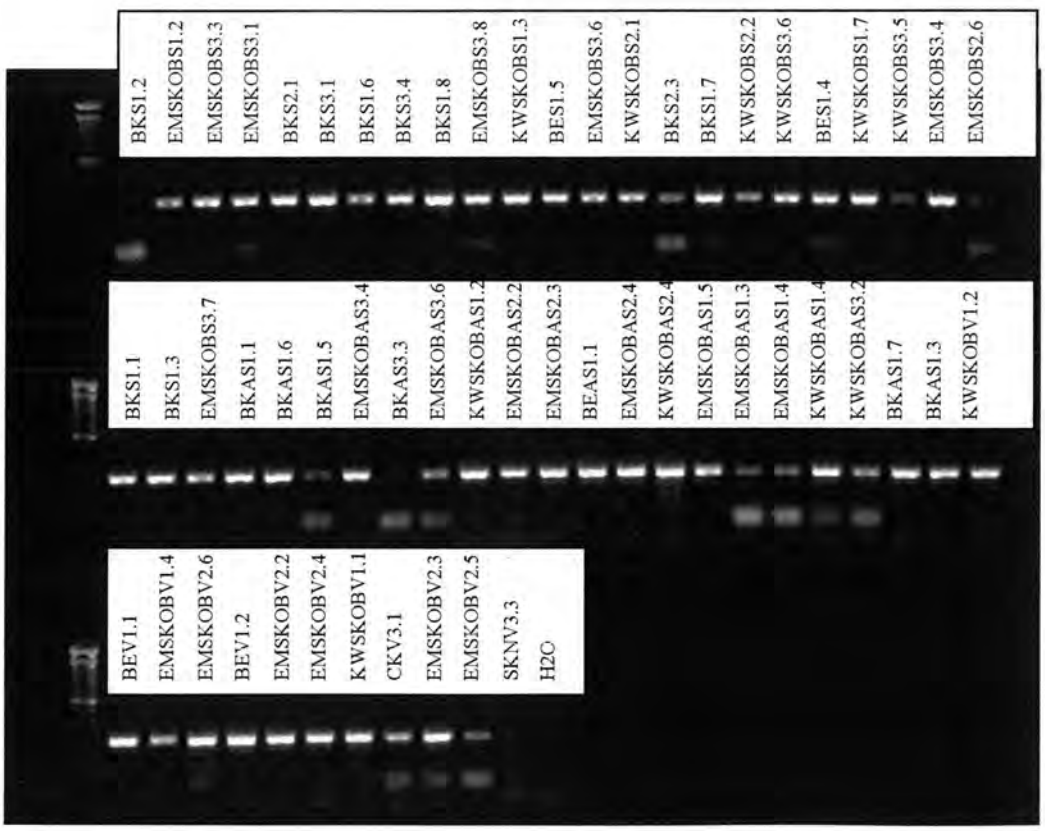
The full coding sequence of human OBCAM was PCR amplified from normal human ovarian surface epithelium RNA and cloned into pcDNA3.1 Zeo (zeocin-resistant)

mammalian expression vector in both the sense and antisense orientations. The OBCAM constructs were transfected into the neomycin-resistant SKOV3 clonal derivative cell line, SKNV3.3 that has no endogenous expression of OBCAM.

4.7.2 Genomic PCR confirms integration of plasmid DNA in transfected cell lines

Using zeocin primers, amplification of a 252bp fragment from DNA made from the transfected cell lines confirmed the presence of integrated plasmid DNA in the majority of the transfectants.

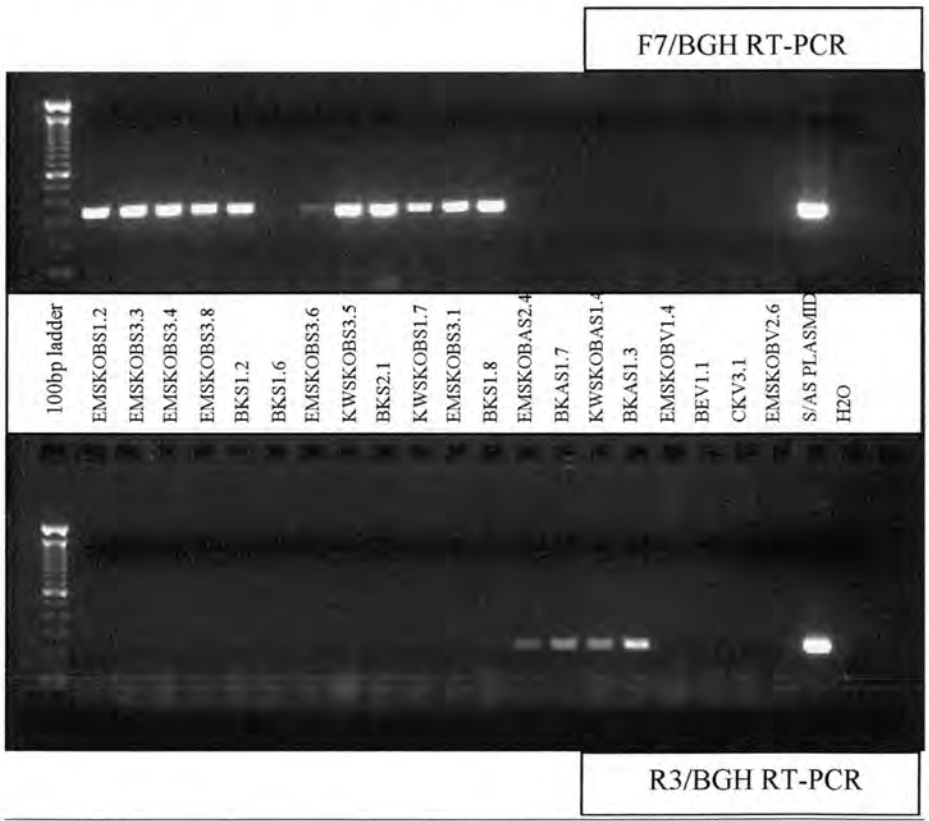
Figure 4.16 Genomic PCR confirms integration of plasmid DNA in the transfected cell lines



4.7.3 RT-PCR determines expression from the transfected constructs

Semi-quantitative RT-PCR was used to determine expression of the sense and antisense constructs in the transfected cell lines. The F7/BGH primer pair was used to detect expression of the sense construct and R3/BGH primer pair used to detect expression of the antisense construct. Several examples of each type of transfected cell line were picked and used in functional assays. Figure 4.17 shows the expression of sense and antisense constructs in the transfected cell lines selected for use in the functional assays. Actin RT-PCR confirmed the integrity of the cDNA at the time of synthesis.

Figure 4.17 Semi-quantitative RT-PCR expression of OBCAM sense and antisense constructs in the SKNV3.3 cell line

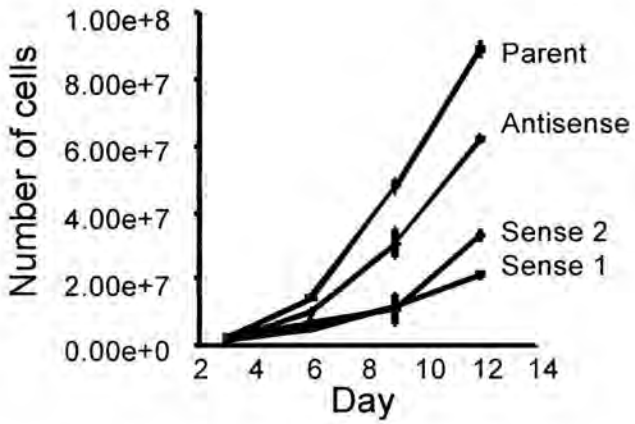


4.8 Functional assays

4.8.1 *In vitro* growth assay

The effect of OBCAM expression was determined in growth assays, comparing parent, OBCAM sense (2 independent clones) and anti-sense transfected clonal SKOV3 cells. Transfection of OBCAM sense expression construct into SKNV3.3 cells resulted in suppressed growth *in vitro* as compared with SKNV3.3 control and OBCAM anti-sense transfected cells. The assay was repeated three times. The data shown in figure 4.18 is from one experiment.

Figure 4.18 *In vitro* growth assay with parent, OBCAM sense and antisense transfected cell lines

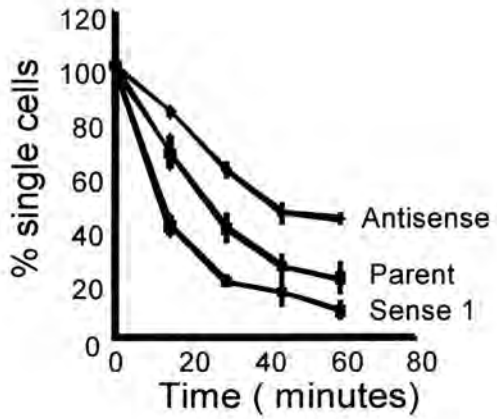


4.8.2 *In vitro* aggregation assay

As a measure of the tendency of cells to aggregate, and therefore of cell adhesion function, the number of single cells remaining in suspensions of parent, OBCAM sense (2 clones) and antisense-transfected clonal SKOV3 cells were counted over a time course. Transfection of OBCAM sense expression construct into SKNV3.3 cells resulted

in enhanced rate of aggregation of cells compared to the parent cells. Transfection of the OBCAM antisense expression construct into SKNV3.3 cells resulted in decreased aggregation of the cells compared to the parent. As there is no endogenous expression of OBCAM in the parent cell line, it may be postulated that the antisense construct is targeting a similar but as yet unidentified member of the IgLON family. The assay was repeated three times and the graph in figure 4.19 shows the mean of the 3 repeats.

Figure 4.19 *In vitro* cell aggregation assay with parent, OBCAM sense and antisense transfected cell lines

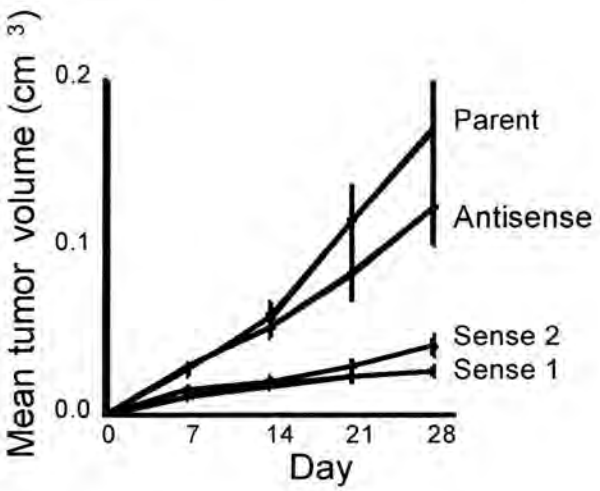


4.8.3 *In vivo* subcutaneous tumour growth assays

Tumour growth was measured over a 28 day period following sub-cutaneous injection of parent, OBCAM sense and antisense-transfected clonal SKOV3 cells into nude mice. Bilateral subcutaneous injections were made into three mice per cell line giving a total of six potential tumours per cell line. The experiment was repeated three times. Figure

4.20 shows representative data from one experiment. Sub-cutaneous tumour growth in nude mice is markedly suppressed by OBCAM expression in SKNV3.3, compared with parent and OBCAM antisense-transfected controls.

Figure 4.20 *In vivo* subcutaneous tumour growth assay with parent, OBCAM sense and antisense transfected cell lines



4.8.4 *In vivo* intra-peritoneal tumour growth assays

To create an intra-peritoneal model of tumour growth, parent, OBCAM sense and antisense-transfected clonal SKOV3 cells were injected intraperitoneally into nude mice. Three mice per cell line were injected. Tumours were visualised at 65 days, in two independent experiments. OBCAM transfection markedly suppressed tumour growth and intra-peritoneal attachment in nude mice, compared with parent and OBCAM antisense-transfected cells. The data shown in figures 4.21 and 4.22 is data from one experiment. The mean tumour weight per mouse was 2634mg for parent and antisense,

and 283mg for the independent sense controls (p=0.0001) and is shown in figure 4.21. Figure 4.22 shows the tumours removed from the 4 groups of mice at the time of their cull.

Figure 4.21 *In vivo* intraperitoneal tumour growth assay – Mean tumour volume per mouse in controls versus sense transfected cell line injected mice

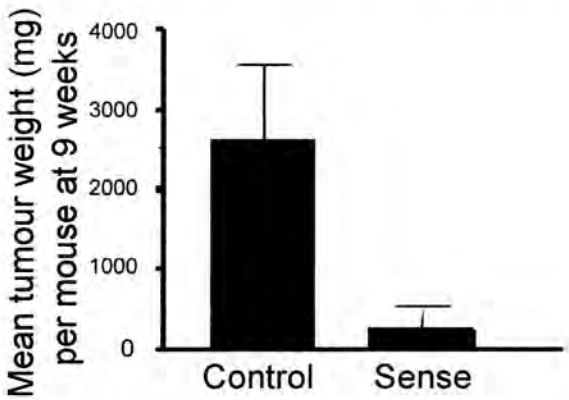
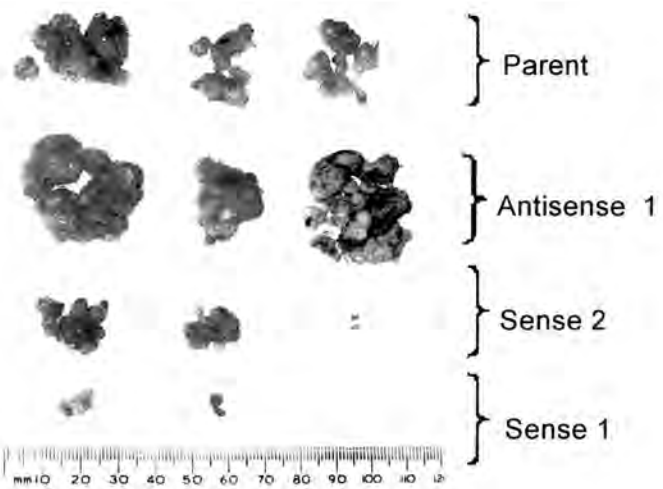


Figure 4.22 *In vivo* intraperitoneal tumour growth assay with parent, OBCAM sense and antisense transfected cell lines



5. RESULTS

INVESTIGATION OF OTHER CANDIDATE TUMOUR SUPPRESSOR GENES FROM THE 11q24-q25 REGION

Although it was clear from early on that the investigation of OBCAM as a candidate tumour suppressor gene associated with the LOH of D11S4085 was going to be fascinating and certainly important, it was felt that the other significant findings of the LOH study should not be overlooked or forgotten. Preliminary investigation of several of these findings was undertaken. Investigation of a gene close to the marker D11S4150 was initiated and analysis of methylation and mutation of the Barx2 gene, another candidate tumour suppressor gene in the region was performed. Bioinformatics analysis was performed in attempt to identify genes close to the marker D11S4131 but due to the unfinished state of sequencing in this region, no candidate genes could be identified. Adverse survival was also associated with LOH at D11S1309. Although established to be the most telomeric marker used in this study the incomplete state of the sequence in this region did not allow identification of genes near this marker.

It must be stressed that the following results are preliminary and sometimes incomplete data but they are included to show the breadth of investigation undertaken in trying to identify candidate tumour suppressor genes from the 11q24-5 region.

5.1 Investigation of Fli1 as a candidate tumour suppressor gene from the 11q24-5 region

In several previous studies, LOH at D11S912 has been found to be associated with adverse survival. No such association was found in this analysis ($p = 0.6489$). Adverse survival was however associated with LOH at D11S4150 ($p = 0.0355$). When this association of adverse survival with LOH at D11S4150 was first identified, a search for candidate genes near this locus was instituted. At this time, the best prediction of

microsatellite order placed D11S4150 and D11S912 next to each other. D11S912 was found to lie within an intron of the Fli1 gene and this gene appeared to lie immediately centromeric of D11S4150. As a result of these findings, it was decided to investigate Fli1 as a candidate tumour suppressor gene.

5.1.1 Expression of Fli1 in normal ovarian surface epithelium and ovarian cancer cell lines

Expression analysis of Fli1 by semi-quantitative RT-PCR was performed on a panel of thirteen ovarian cancer cell lines and a sample of normal human ovarian surface epithelium. Fli1 was found to be strongly expressed in normal ovarian surface epithelium. Expression of Fli1 equivalent to that in normal ovarian surface epithelium was found in 7/13 (54%) of the cell lines with 6/13 (46%) cell lines displaying markedly reduced or absent expression of Fli1. This data is shown in figure 5.1.

5.1.2 Expression of Fli1 in human ovarian tumours

RT-PCR using the same primer combination as used to determine expression in normal ovary and the cell lines was used to investigate the expression of Fli1 in a panel of thirty human ovarian tumours. Figure 5.2 shows that strong expression of Fli1 was found in every tumour sample. Having found that FLI1 is expressed in only approximately 50% of ovarian cancer cell lines this was an unexpected result. These ovarian tumour samples were non-microdissected and would be expected to contain some normal, non-malignant tissue such as blood vessels and stromal tissue. It is known that Fli1 is expressed in

haematopoeitic tissues and it is therefore likely that the observed Fli1 expression is a reflection of contaminating normal tissue in the ovarian tumour samples.

Figure 5.1 Semi-quantitative RT-PCR expression of Fli1 in ovarian cancer cell lines

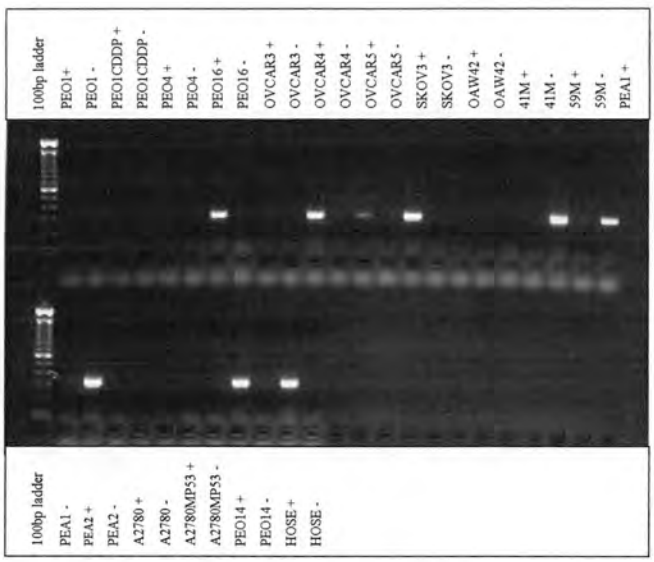
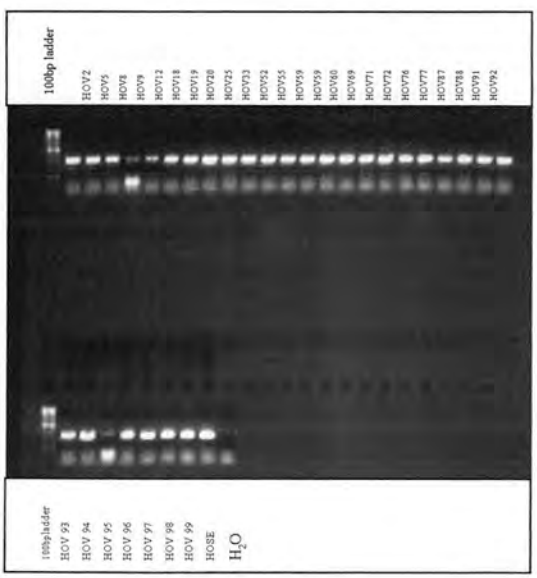


Figure 5.2 Semi-quantitative RT-PCR expression of Fli in human ovarian tumours



5.1.3 Identification of the Fli1 CpG island and determination of its methylation status

A 780bp putative CpG island for Fli1 was identified on BAC AP001535.2 (nucleotides 176084-176863). This was identified by performing NIX analysis on this BAC that was known to contain FLI1 gene sequence. The GRAIL CpG island prediction algorithm within the NIX analysis produced a good quality CpG island prediction. This CpG island was approximately 1.8kb upstream of the gene.

Methylation Specific PCR (MSP) primers were designed to distinguish methylated (M) from unmethylated (U) alleles of the Fli1 CpG island sequence following sodium bisulphite modification of genomic DNA. An MSP assay that amplified a 211bp fragment was used to determine the methylation status of the Fli-1 CpG island.

5.1.3.1 The Fli1 CpG Island is Unmethylated in Normal Human Ovary

MSP of bisulphite-treated genomic DNAs extracted from normal ovaries from 2 women who underwent oophorectomy for non-malignant conditions showed that the Fli1 CpG island is normally unmethylated. Bisulphite sequencing across the 211bp Fli1 MS-PCR product from these normal ovaries showed sequence that was unmethylated at all but one of the 13 CpGs within this region.

5.1.3.2 Fli1 methylation in primary ovarian tumours

MSP was performed to detect methylated and unmethylated alleles of Fli1 in 31 ovarian matched normal/tumour DNA pairs. The Fli1 CpG island is somatically methylated in 61% (19/31cases) of ovarian primary tumours. Strong methylation was seen in 8/31 cases and weak methylation in 11/31 cases.

5.1.3.3 Fli1 CpG Island methylation in ovarian cancer cell lines

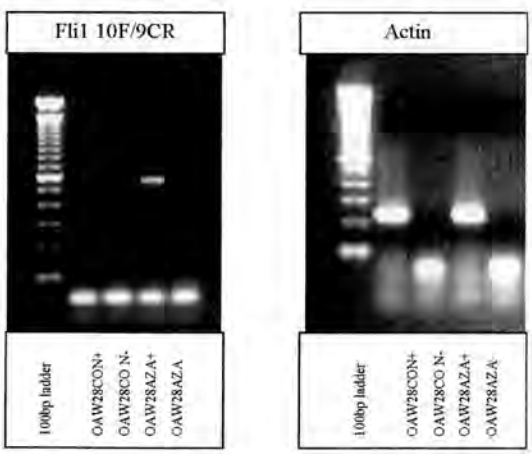
The Fli1 CpG island was found by MSP to be methylated in 67% (12/18) ovarian cancer cell lines.

5.1.3.4 Demethylation and re-expression of Fli1 in OAW28

In order to confirm that methylation was the mechanism underlying the repression of Fli1 expression, OAW28 ovarian cancer cells were exposed to the demethylating agent 5'-aza-2'-deoxycytidine. This cell line does not express Fli1 as determined by semi-quantitative RT-PCR.

Re-expression of Fli1 is clearly evident in the treated cells. In contrast, no Fli1 expression is detectable in control cells (Fig5.4). Actin RT-PCR confirmed the integrity of the isolated RNA and 1st strand cDNA.

Figure 5.4 Re-expression of Fli1 following deoxyazacytidine exposure



5.1.4 Mutation screening of the Fli1 gene using SSCPE

SSCPE was employed as a method for the identification of somatic mutations within the coding region of the Fli1 gene. Primer pairs were designed for each of the 9 exons of Fli1, amplifying from flanking introns across the exons. Four overlapping SSCPE primer pairs for exon 9 were required to allow complete analysis of this exon. SSCPE analysis of 24 normal/tumour DNA pairs was completed for exons 2, 3, 4, 5, 6, 7 and 3 out of 4 of the pairs for exon 9. These 24 pairs were selected because they showed LOH at either or both of D11S4150 and D11S912. It was hoped that by analysing a panel of pairs that had already sustained one "hit" of the gene that this may enhance the chance of finding a mutation. Exons 5 and 6 were analysed in 16 ovarian cancer cell lines. At this time point the study was stopped as newly released data from the HGP suggested that D11S4150 was not so intimately associated with the Fli1 gene as had been previously thought. Of the analysis undertaken only one silent mutation was identified in a single ovarian tumour. Table 5.1 documents this mutation. The alteration was substitution of a guanine nucleotide for an adenine in the third base of codon 210.This did not alter the glutamine aminoacid residue at this position.

Table 5.1 Silent mutation identified in the Fli1 gene

BT pair	Mutation	Nucleotide NM_002017.2	Codon	Aminoacid
(97,98)	A -> G	802	210	Glutamine

5.2 Investigation of BARX2 as a candidate tumour suppressor gene from the 11q24-5 region

Prior to the LOH study in this thesis being performed, a gene called Barx2 had been identified as a candidate tumour suppressor gene from this region. Work in our lab had suggested that Barx2 had tumour suppressor characteristics. In order to identify a second hit for this gene, methylation and mutation studies were undertaken.

5.2.1The Barx2 CpG island is not methylated in ovarian tumours or ovarian cancer cell lines

Sequence of the Barx2 CpG island was published by Hjalt and Murray and primers were designed to a region of this island. Methylation specific polymerase chain reaction was used to amplify a 194bp fragment from bisulphite treated DNA extracted from 25 cancer cellines and 12 normal/ovarian tumour pairs from patients with epithelial ovarian cancer. Not one of the sixteen ovarian cancer cell lines showed evidence of methylation of the Barx2 CpG island. Of the nine non-ovarian cancer cell lines, K562 and Hela were both methylated. There was no evidence of somatic methylation of the Barx2 CpG island; none of the 12 matched normal/ tumour pairs showed evidence of methylation.

5.2.2 Two Barx2 mis sense mutations are identified in a panel of cancer cell lines

Two Barx2 mis-sense mutations were identified in the panel of 71 cancer cell lines comprising 16 ovarian cancer cell lines and 56 non ovarian cancer cell lines. It is not known whether these represent somatic changes. The first mutation identified shows a heterozygous peak corresponding to the presence of both T and a C nucleotide at

position 142 (AF171220) in three ovarian cancer cell lines (SKOV3, PEO14 and PEO23) and one cell line derived from a malignant melanoma (DX3). This alteration results in the substitution of a hydrophilic neutral serine residue for a hydrophobic proline residue at codon 39, contained within exon 2 of Barx2. The second mutation, identified in the chronic myelogenous leukaemia cell line K562, also shows a heterozygous peak, corresponding to the presence of both guanine and cytosine nucleotides at position 217 (AF171220). This results in the substitution of a hydrophobic alanine residue for a hydrophobic proline residue at codon 63, also in the second exon of BARX2.

No mutations were identified in the panel of 70 normal/ovarian paired DNAs.

Three single nucleotide polymorphisms (SNPs) in Barx2 have been described. Two of these SNPs were identified in this analysis. Two normal/ovarian tumour pairs were homozygous for the C->T SNP in exon 4 at nucleotide 400. Three pairs were heterozygous at this position with one of these pairs showing reduction to homozgosity in the tumour suggesting that the tumour had been subject to LOH of one allele. The same pattern was observed for these five pairs with the C->T SNP in intron 3 of Barx2 at nucleotide 360. Table 5.2 shows the Barx2 mutations identified.

Table 5.2 Mis-sense mutations identified in the Barx2 gene

Exon	Mutation	Nucleotide AF171220	Codon	Aminoacid	Cell lines
2	T -> C	142	39	Serine -> Proline	DX3 SKOV3 PEO14 PEO23
2	G -> C	217	63	Alanine -> Proline	K562

6. DISCUSSION

6.1 Preamble

The starting point for the work in this thesis was the finding of several independent studies showing a high frequency of LOH in the 11q24-5 region in epithelial ovarian cancer. (Foulkes et al. 1993) (Gabra et al. 1995) (Gabra et al. 1996) (Davis et al. 1996). This suggested that this region houses a tumour suppressor gene involved in this disease. For those women unfortunate enough to be diagnosed with epithelial ovarian cancer, the overall five year survival rate is poor. Our understanding of the aetiology and pathogenesis of ovarian cancer is limited and with the exception of platinum and taxane compounds, there have been few new developments in the therapeutic management of ovarian cancer over the last forty years. The quest therefore, for greater knowledge and understanding of this disease, which may lead to earlier diagnosis and more effective treatment is ongoing. Identification of genes involved in the genesis of epithelial ovarian cancer is the first step towards this goal. Working in the setting of the multidisciplinary gynaeoncology clinic, the availability of clinical resources allied with comprehensive clinicopathological and follow-up data for these specimens has provided an invaluable opportunity to investigate the genetic basis of ovarian cancer. Understanding the role of tumour suppressor genes in cancer is crucial and characterisation of these genes is a vital key to novel concepts in malignant disease.

6.2 Tumour suppressor genes

The definition of a tumour suppressor gene is a hotly debated topic. The earliest description of tumour suppressor genes came from Theodor Boveri in 1902 when he postulated the existence of negative regulators of tumour development. Some seventy

years later, Alfred Knudson proposed that development of the childhood tumour retinoblastoma required two rate limiting genetic hits, subsequently shown to represent mutations of both alleles of a tumour suppressor gene (Knudson 1971). The requirement for two genetic hits established that these genes act recessively at the cellular level. The link between germline mutation and increased cancer risk provides incontrovertible evidence of a gene's role in tumourigenesis. Positional cloning methods based on human cancer predisposition syndromes have identified several tumour suppressor genes including RB (Friend et al. 1987), WT1 (Call et al. 1990) (Gessler et al. 1990), APC (Hampton et al. 1991), NF1 (Cawthon et al. 1990) (White and O'Connell 1991), NF2 (Rouleau et al. 1993) and VHL (Latif et al. 1993). In sporadic cancers, LOH analysis has been used as a method to identify regions of the genome that may harbour tumour suppressor genes.

The identification of the presence of truncating or inactivating mutations in a candidate tumour suppressor gene has until now been regarded as necessary to designate a gene as a tumour suppressor gene. Haber and Harlow (Haber and Harlow 1997) define tumour suppressor genes as " genes that sustain loss of function mutations in the development of cancer". They believe that demonstration of LOH in the region of a candidate tumour suppressor gene with concomitant reduction of expression of that gene in the same tumour type is not adequate proof of it being a tumour suppressor gene. Down-regulation of gene expression may simply be a reflection of the malignant phenotype rather than a causal factor in cancer development. The demonstration of gene inactivating mutations in DNA from tumours suggests that these events provide a growth advantage to the cell and are thereby selected for. More recently however, the

importance of epigenetic regulation as a method of tumour suppressor gene inactivation has been highlighted. Baylin and Herman (Baylin and Herman 2000) believe that methylation acts as an alternative to mutations to disrupt tumour suppressor gene function. Studies demonstrating the ability of treatment with a demethylating agent to promote transcriptional reactivation of silenced genes provide evidence that CpG island methylation is an active process that provides a growth advantage to the cell that is selected for.

The functions of the known tumour suppressor genes are diverse, ranging from DNA-binding transcription factors (p53) to cell cycle kinase inhibitors (p16) to genes involved in DNA repair (MSH2, MLH1, BRCA1 And BRCA2). Considered as a group, tumour suppressor genes are integral components of complex cellular pathways regulating cell proliferation, differentiation, apoptosis and response to genetic damage. Functional experiments using transgenic and knockout mice have been used to demonstrate tumour suppressor gene function in vivo (Hooper 1998). In vitro gene transfection studies of candidate tumour suppressor genes have been criticised for producing non-physiological levels of gene expression and possibly artefactual phenotypes. Furthermore, transfection studies are also subject to clonal variation.

6.3 LOH analysis

Although LOH studies have been widely used to define regions of chromosomal loss in sporadic cancers, the identification of the associated tumour suppressor genes has proven to be challenging. This approach often highlights many potential candidate genes in a region of LOH, which then require to be individually assessed. Alternatively, lack of

genomic information in the region of loss requires a comprehensive gene identification screen to be initiated. Recent refinement of the human genome sequence has provided an opportunity to re-evaluate the power of the LOH approach for identification of tumour suppressor genes. Previous studies have indicated that a tumour suppressor could reside at 11q24-25 in sporadic ovarian cancer, but candidates until now have remained elusive. During this period of research, data generated by the human genome project has been used to order markers and genes across the 11q24-25 region.

My analysis of LOH in epithelial ovarian cancer on chromosome 11q24-25 is the largest study to date of LOH in this region combining the highest number of cases with the greatest number of microsatellite markers. This has allowed a greatly refined map of LOH on 11q24-25 in epithelial ovarian cancer to be produced.

A 1Mb minimal region of loss centred on D11S4085 has been defined. Additionally, correlation of LOH at individual markers with survival times and the clinicopathological variables of the disease has identified several markers for which LOH is associated with adverse survival. Subsequent multivariate analysis has identified LOH of the marker D11S4131 as an independent adverse prognostic variable. These data suggest the 11q24-25 region houses several potential tumour suppressor genes. Previous LOH studies have produced conflicting results concerning the association of LOH and survival in this region. These conflicting results may be explained by the most recent findings suggesting the presence of several potential tumour suppressor genes in the 11q24-25 region. This highlights the difficulty in attempting to assimilate data from independent LOH studies that have used different and varying numbers of microsatellite markers.

6.4 Candidate tumour suppressor genes from the 11q24-5 region

6.4.1 OBCAM

6.4.1.1 OBCAM – an extracellular protein

The marker with the highest frequency of LOH, D11S4085 lies within the second intron of the gene encoding OBCAM. OBCAM is a 345 amino acid protein that is located entirely extracellularly and is attached to the outer leaflet of the apical bilayer membrane by a GPI anchor. As a member of the immunoglobulin superfamily it is easy to understand how it acts as a cell adhesion molecule. Due to its lack of a transmembrane or cytoplasmic region, it is harder to envisage how this protein can mediate intracellular signalling and act as a tumour suppressor gene. OBCAM mediated signalling may arise as a consequence of heterophilic interactions with proteins on the same cell or on a different cell. Homophilic interaction of OBCAM may induce growth regulatory signalling indirectly by bringing ligand and receptors into close enough proximity to allow their interaction. However, this mechanism whereby OBCAM could influence signalling would be just as likely to oppose activating ligands and receptors.

Heterophilic interaction of OBCAM with a transmembrane protein in the same cell may modulate cell signalling. This may be a positive or negative regulation. Binding of OBCAM to a growth factor receptor may act as a competitive antagonist by inhibiting access of the ligand to its receptor. Alternatively, binding of OBCAM to a growth factor receptor may enhance its activation leading to enhanced growth regulation. GPI-anchorage to the cell membrane facilitates diffusion of these proteins in the plane of the cell membrane. GPI-anchored proteins tend to localise in the cholesterol rich areas of the cell membrane known as lipid rafts (Friedrichson and Kurzchalia 1998) where they can

interact with other similarly localised proteins. Consequently, a potential function of rafts is to provide a local concentration of receptors to facilitate rapid signal transduction as well as limiting potential cross talk between signalling pathways. The GPI-anchored neuronal cell adhesion molecule contactin/F11 has been shown to localise with the src family protein tyrosine kinase, Fyn in detergent-resistant immune complexes (Zisch et al. 1995). This tyrosine kinase is associated with the inner leaflet of the plasma membrane. The authors propose that signalling events underlying dynamic interactions via GPI-linked contactin/F11 in the nervous system rely, at least partially on the action of the nonreceptor tyrosine kinase Fyn. GP55, a member of the IgLON family in the chicken, can inhibit neurite extension on substrata. This process can be inhibited by pertussis toxin, indicating that IgLON molecules may function via a G-protein coupled pathway (Clarke and Moss 1997).

6.4.1.2 Mechanisms of OBCAM inactivation in ovarian cancer

The frequency of OBCAM somatic mutation is extremely low with only three mis-sense mutations in a total of 328 primary tumour samples and cell lines being identified. No truncating mutations in primary epithelial ovarian cancers were identified but a potential loss of function somatic mutation has been investigated. In contrast to its low somatic mutation rate, the frequency of OBCAM somatic methylation is extremely high (83%). If one accepts the broader definition of a tumour suppressor gene that acknowledges the contribution of methylation to gene inactivation then OBCAM clearly undergoes inactivation in keeping with Knudson's two hit mechanism for loss of tumour suppressor gene function. OBCAM is subject to frequent LOH and somatic methylation in the vast

majority of ovarian tumours. The observed somatic methylation of OBCAM appears to be specifically selected for with 90% of those cases with LOH displaying somatic methylation whilst only 69% of cases without LOH are methylated. Barx2, a gene located centromeric of OBCAM in the 11q24 region is not methylated in ovarian tumours. This suggests that methylation in ovarian tumours specifically targets OBCAM rather than merely being the result of a chromosome wide or global phenomenon resulting from the malignant phenotype. Furthermore, the ability of treatment with deoxyazacytidine to re-express OBCAM in a non-expressing ovarian cancer cell line that has extensive methylation of its CpG island suggests that methylation is the gene inactivating mechanism and not just a bystander effect.

Although only one somatic mis-sense mutation (P95R) in a primary tumor sample was detected in a series comprising 212 ovarian tumours, impaired protein function may well result from the non-conservative change altering a proline to an arginine residue. A collaboration with Paul Bates (CRUK, Biomolecular Modelling Laboratory) has provided some insight into the potential significance of the identified somatic mis-sense mutation. The three-dimensional model of the first Ig domain that was generated, whilst not sufficiently accurate to determine if this domain actually is involved in homophilic adhesion (i.e. by constructing protein-protein docked models), does not preclude this possibility. The BED face of the domain has been implicated in protein-protein interactions between molecules on the same cell, such as for ICAM-1 (Casasnovas, Bickford, and Springer 1998). The opposite face of the domain, the CFG face, often mediates homo- and heterophilic protein interactions between cells (Chothia and Jones 1997) and has been proposed to have evolved as a sticky patch to recognise a variety of

protein-protein interactions (Springer 1990). A model for a related GPI linked molecule to OBCAM, carcinoembryonic antigen (Bates, Luo, and Sternberg 1992) suggests that by utilising the BED face for interactions on the same cell and the GCF face of the first N-terminal domains for homophilic adhesion, a distributed network of interactions can exist between cells. A similar network has been shown within the crystal structure of N-cadherin (Shapiro et al. 1995). Therefore, it is possible the P95R mutation, lying between the D and E strands, could potentially disrupt a similar interaction between OBCAM molecules on the same cell and, therefore, indirectly affect potential interactions between cells. A less likely but possible explanation is that P95 is located within the fold of the D and E strands, that a change to a hydrophilic arginine residue could disrupt this fold, and therefore OBCAM protein-protein interactions.

6.4.1.3 Evolutionary conservation of OBCAM

The first member of the IgLON family of proteins to be identified was LAMP. LAMP was initially identified in the rat limbic system (Levitt 1984). Since their identification in the nervous system, the work on this family of proteins has concentrated almost exclusively on their role in the developing nervous system. Although three human IgLON molecules have been cloned, their role in Homo sapiens is implicated solely from studies of homologues in chickens and rats. Even in chickens and rats, little infact is known about the expression and function of IgLONs outside the nervous system. In the published literature, there is only one study documenting investigation of IgLON expression out with the nervous system. Hachisuka et al (Hachisuka et al. 1996) developed monoclonal antibodies to a synthetic OBCAM peptide and documented high

levels of OBCAM expression in bovine, rat, mouse, guinea pig and rabbit brains. In other tissues, the protein was found in the spleen at very low levels but not at all in the liver or kidney of the rat.

The interest in the IgLONs in the nervous system has focussed on their role in the development of the nervous system. The finding of highly specific and temporal expression of OBCAM, NTM and LAMP in the developing nervous system is evidence supportive of a role in controlling neurite outgrowth and axonal guidance. Indeed, LAMP and NTM have been shown to have bifunctional effects on neurite outgrowth that are neuron type-specific and strongly suggest that homophilic interactions promote outgrowth, whereas heterophilic interactions between NTM and LAMP inhibit neurite outgrowth (Gil et al. 1998). The ability of OBCAM to modify neurite outgrowth has not yet been studied. A recent publication by McNamee et al (McNamee et al. 2002) suggests that IgLONs may not have a primary role in axon guidance but may be more important for cell to cell adhesion and recognition. These authors speculate that clustering of IgLONs on the cell surface may be more important for the stability of interactions and the initiation of the appropriate signal transduction cascade.

The high degree of conservation of OBCAM between species is strong evidence for a protein with a fundamentally important function. The homology is greatest amongst mammalian species with RN being the closest homologue to HS OBCAM. As well as looking at the overall homology between species and the other human IgLONs it is interesting to look for conserved regions of the protein. The 100% homology between the first 60 amino acids of NTM and OBCAM (also 100% conserved at nucleotide level) suggests a strong evolutionary constraint inhibiting divergence. It is interesting to note

that at both the amino acid and the nucleotide level, the position of the three mis-sense mutations are conserved between mammalian species. In fact, the position of the third mis-sense mutation is conserved not only between species but between IgLON family members. This provides evidence that the presence of any of these three mutations would be deleterious to OBCAM protein function. The high conservation of this protein suggests that OBCAM plays a fundamental physiological role.

6.4.1.4 OBCAM and the ovary

In functional analyses, OBCAM expression in a clonal derivative of SKOV3 suppressed growth and enhanced cell-cell aggregation *in vitro*; and also suppressed sub-cutaneous tumour growth and intra-peritoneal tumour growth *in vivo*. These features suggested both tumour suppressor and cell-cell adhesion functions for OBCAM. The finding that a GPI-anchored molecule has the characteristics of a candidate tumour suppressor gene is unusual. The only other GPI-anchored molecule for which tumor suppressor activity has been proposed is hyaluronidase 2 (HYAL2) (Rai et al. 2001). *HYAL2* was proposed as a candidate lung tumor suppressor gene based upon its localisation to 3p21.3 (Rai et al 2001), a region of chromosomal deletion in lung and breast cancers (Wei et al. 1996) (Lerman and Minna 2000). No functional evidence has been presented to support a tumour suppressor function for HYAL2. Indeed, it is now been demonstrated to function as the receptor for the jaagsiekte sheep retrovirus, the envelope protein of which mediates oncogenic transformation (Rai et al. 2001).

It has been proposed that "incessant ovulation" contributes a major factor underlying ovarian epithelial oncogenesis (Fathalla 1971a). Ovulation has been considered to be a

cyclical inflammatory reaction (Espey 1980) (Ness and Cottreau 1999), with the involvement of multiple proinflammatory cytokines, including interleukin 1-beta (IL-1β), its associated type I and type II receptors and receptor antagonist (Kol, Kehat, and Adashi 2002). IL-1β appears to promote various facets of the ovulatory process by stimulating the expression of numerous downstream effector genes in a tightly controlled hierarchical manner (Ando et al. 1998). In a chondrosarcoma model of arthritis, OBCAM expression in chondrocytes was downregulated 4-fold in response to IL-1β exposure (Vincenti and Brinckerhoff 2001). As the ovarian surface epithelium is a site of localised and tightly regulated IL-1β production during the "tear and repair" cycle of ovulation, it is possible that OBCAM expression may be subject to modulation during this process. This leads to the proposal that continual cyclical down-regulation of OBCAM is a contributing factor to the early steps of ovarian carcinogenesis. A speculative but attractive model inferred from the functional data would be that loss of OBCAM would reduce intercellular adhesion and accelerate cell growth simultaneously, thereby promoting the early steps of ovarian carcinogenesis.

6.4.2 Fli1

In several studies, LOH at D11S912 has been found to be associated with adverse survival. This finding was not corroborated by the present analysis. D11S912 lies within the Fli1 gene. Fli1 is a member of the Ets family of transcription factors that are characterised by the presence of a highly conserved DNA binding domain called the Ets domain. Most Ets proteins have been shown to be transcriptional activators but there is

also evidence that some of these genes act as transcriptional repressors (Mavrothalassitis and Ghysdael 2000).

Fli1 was first identified as a common site of retroviral integration in friend virus-induced erythroleukaemias (Ben-David et al. 1991). In cancer, Fli1 is widely regarded as an oncogene and therefore the suggestion that Fli1 may have tumour suppressor function albeit contextually in the setting of ovarian cancer would radically challenge current thinking about the function of this gene. There is a spectrum of cancer including soft tissue sarcomas and leukaemias in which balanced reciprocal translocations result in the production of oncogenic fusion proteins that contain the Ets DNA binding domain. Loss of function of Ets family members may also contribute to the pathogenesis of human cancers. In TEL/AML leukaemias there is often deletion of the residual TEL allele resulting in there being no functional TEL in the cell suggesting that TEL may have tumour suppressor function.

Fli1 is subject to a translocation in human Ewing's tumour. More than 85% of Ewing's family tumours carry a specific chromosomal translocation that fuses the amino terminus of the EWS gene to the carboxy terminus of the Fli1 gene. The product of this translocation is a potent transforming oncogene. The mechanisms by which the EWS/Fli1 protein exerts its oncogenic actions are currently being investigated by the scientific community. Repression of the gene encoding TGFbetaRII appears to be a major target of the EWS/Fli1 oncoprotein (Hahm et al. 1999). In normal cells, Fli1 promotes transcription of TGFbetaRII (Truong and Ben-David 2000). In this manner, Fli1 may exert tumour suppressor properties. Loss of Fli1 function with subsequent

down regulation of the TGFbetaRII expression would be expected to be associated with a loss of growth inhibition normally imposed by TGFbeta signalling.

Analysis in the lab has shown the normal ovarian surface epithelium to be a site of strong Fli1 expression. In a panel of ovarian cancer cell lines, Fli1 was expressed at levels equivalent to that of normal HOSE in 7/13 ovarian cancer cell lines but its expression was markedly reduced or absent in the remaining lines. The gene was somatically methylated in 19/31 human ovarian tumours and methylated in 12/18 ovarian cancer cell lines. Treatment of an ovarian cancer cell line that has no expression of Fli1with deoxyazacytidine resulted in re-expression of Fli1 suggesting that methylation is the method of gene silencing in this cell line. An analysis of the Fli1 gene by SSCP did not detect any mis-sense or inactivating mutations of the gene. This analysis was not fully completed for exons 1, 8 and 9 and the number of samples was small. Despite this however, having detected a lower second hit rate of methylation (61% Fli1 v 83% OBCAM) than in the OBCAM gene it seems reasonable to surmise that if Fli1 was a bona fide tumour suppressor gene we would probably have found an inactivating mutation in the samples tested.

6.4.3 P53AIP1 and D11S4150

To date, there are no published data regarding LOH at D11S4150 in epithelial ovarian cancer. In this analysis, LOH at D11S4150 was found to be associated with adverse survival. 76bp centromeric of D11S4150 lies KCNJ5, a gene encoding a potassium channel protein, which is an unlikely candidate tumour suppressor gene. 134bp telomeric of D11S4150 lies p53- regulated apoptosis-inducing protein1 (p53AIP1). As

its name suggests, p53AIP1 is a downstream target of p53. p53AIP1 was identified by direct cloning of p53 binding sequences from human genomic DNA (Oda et al. 2000). It has been suggested that p53AIP1 is likely to play an important role in mediating p53 dependent apoptosis with phosporylation of Serine-46 of p53 appearing to regulate the transcriptional activation of p53AIP1. In colony forming assays, the same authors show that p53AIP1 functions as a growth suppressor. p53AIP1 is thus a good candidate tumour suppressor gene. p53 mutation in ovarian cancer is not a predictor of outcome. It may be that knowledge of the status of both p53 and p53AIP1 in tumours may give a clearer indication of the functional status of the p53 pathway and thereby be of use as a prognostic indicator.

6.5 Summary and future directions

The LOH study presented in this thesis has produced several significant findings that have led to the identification of at least one very exciting candidate tumour suppressor gene from the 11q24-5 region in epithelial ovarian cancer. Information made freely available through the Human Genome Project has transformed the power of LOH analyses and has contributed enormously to the success of this project.

The data presented provides strong evidence that inactivation of the extracellular cell adhesion molecule OBCAM is an early event in ovarian carcinogenesis, and that it is an excellent candidate for the 11q25 epithelial ovarian cancer tumour suppressor gene.

The finding of adverse survival of patients with LOH at D11S4150 and D11S4131 suggests the presence of genes important in ovarian cancer near these loci. p53AIP1 is an excellent candidate tumour suppressor gene at the D11S4150 locus and investigation

of this gene in ovarian cancer must be pursued. As yet there are no annotated genes close to the D11S4131 locus whose loss is an independent adverse prognostic variable in epithelial ovarian cancer. At the present time therefore, expression analysis of ESTs in the vicinity of D11S4131 may be a reasonable first step approach to cloning this important gene.

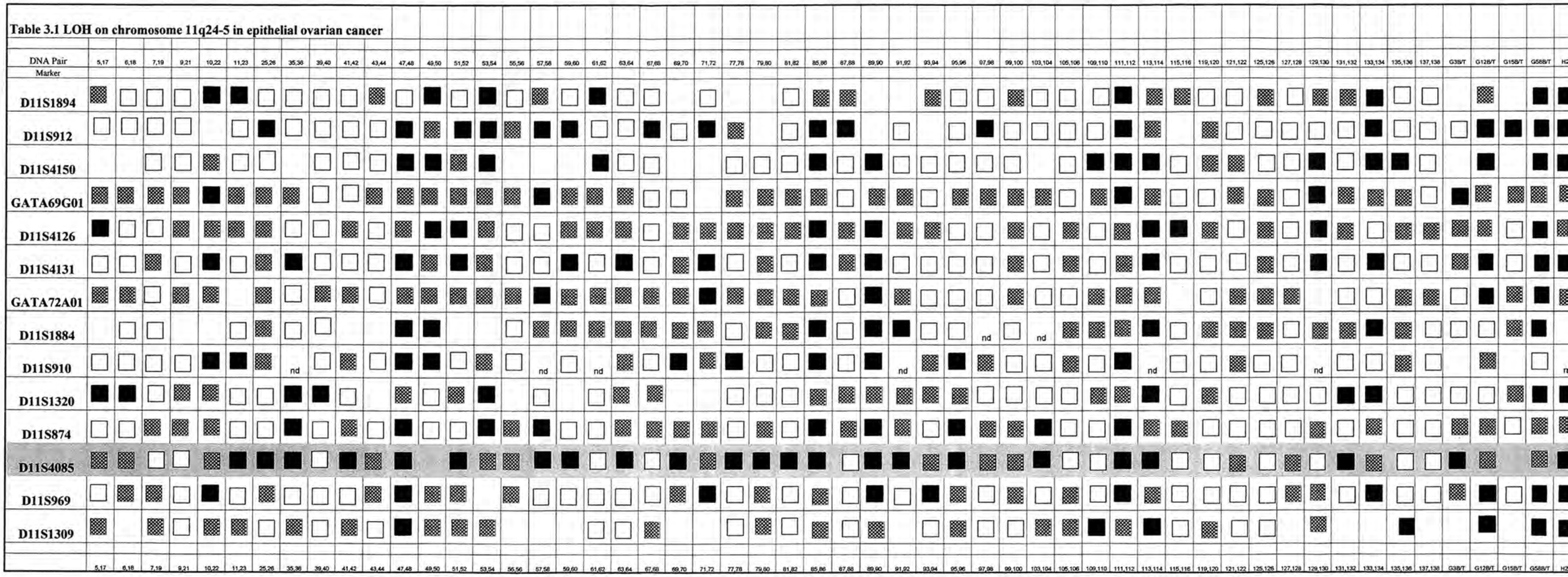
An important area for future studies investigating the OBCAM gene will be to study the signal transduction biology of OBCAM. The nature of OBCAM's signalling pathway and its transcriptional effects have yet to be elucidated. In order to characterise the signalling pathway(s) through which expression of OBCAM-induced genes is mediated, Western blotting studies using protein isolates from OBCAM transfected and control cells can be performed with a range of antibodies against major signalling pathway components. Creation of a Tet-On doxycycline inducible transfection system would be a valuable resource for analysing the effects of regulated OBCAM expression and would provide the ideal resource to investigate the transcriptional consequences of OBCAM expression in a genome-wide profiling study. In view of the lack of somatic mutation of OBCAM in ovarian tumours, creation of a mouse knockout of OBCAM will hopefully confirm its tumour suppressor function. Inactivation of OBCAM appears to occur early in ovarian carcinogenesis. Unlike in other cancers such as cervical and colorectal tumours, it is uncertain whether or not there is an ovarian cancer precursor lesion. It will be intriguing to investigate whether OBCAM inactivation also occurs in borderline and or benign ovarian tumours. The potential for identification of methylated OBCAM in the blood of patients with ovarian cancer may provide the basis for a diagnostic test for ovarian cancer. Furthermore, following identification of a phenotype associated with

OBCAM expression it should be possible to identify OBCAM mimetic molecules for use in therapy of patients with ovarian cancer. This is the first description of the involvement of IgLON family members in cancer and as such opens up a whole new area of research.

The quest to understand the molecular biology of epithelial ovarian cancer with the ultimate aim of translating this knowledge into methods for achieving earlier detection and better treatment for patients with ovarian cancer is an evolving area of research. Data from the Human Genome Project and rapidly developing powerful new technologies are providing new levels of investigation. Hopefully in the not too distant future we will see that research such as that in this thesis will have lead to developments for use in the clinic.

7. APPENDIX

Table 3.1 LOH on chromosome 11q24-5 in epithelial ovarian cancer



H308	H386	H311	H393	H389	H307	H269	H303	H300	H295	273	397	401	417	423	432	449	453	474	481	2	14	16	17	21	24	26	29	30	31	34	35	43	45	46	47	48	53	55	56	57	60	69	75	77	296	380	429	487	491	496	497	524	538	543	545	546
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	nd																																																						
H308	H386	H311	H393	H389	H307	H269	H303	H300	H295	273	397	401	417	423	432	449	453	474	481	2	14	16	17	21	24	26	29	30	31	34	35	43	45	46	47	48	53	55	56	57	60	69	75	77	296	380	429	487	491	496	497	524	538	543	545	546

Figure 4.11 Clustalx analysis of amino acid homology of OBCAM between species

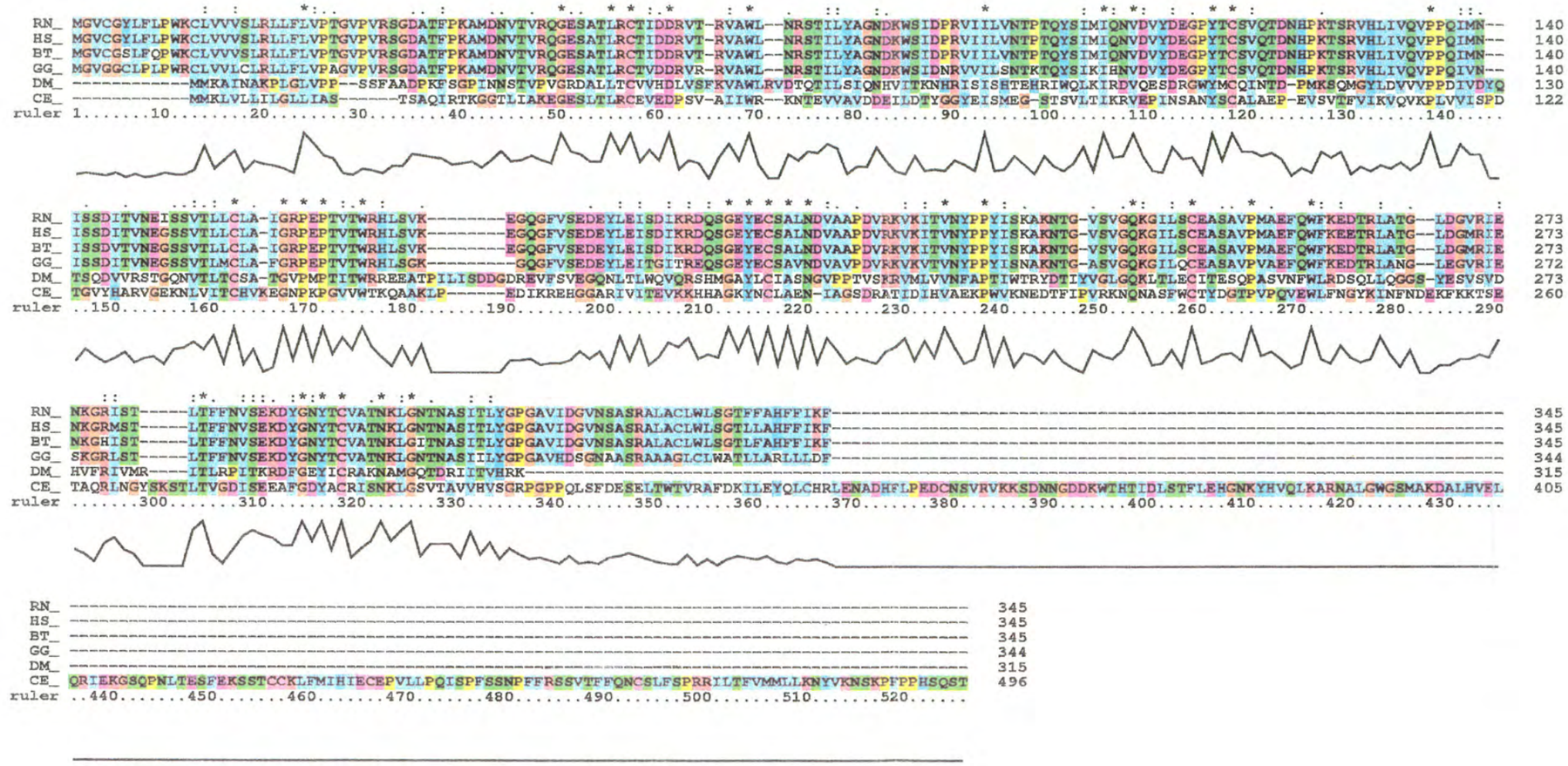


Figure 4.12 Clustalx analysis of nucleotide homology of OBCAM between species

Figure 4.14 Clustalx analysis of amino acid homology of OBCAM and the other human IgLONS

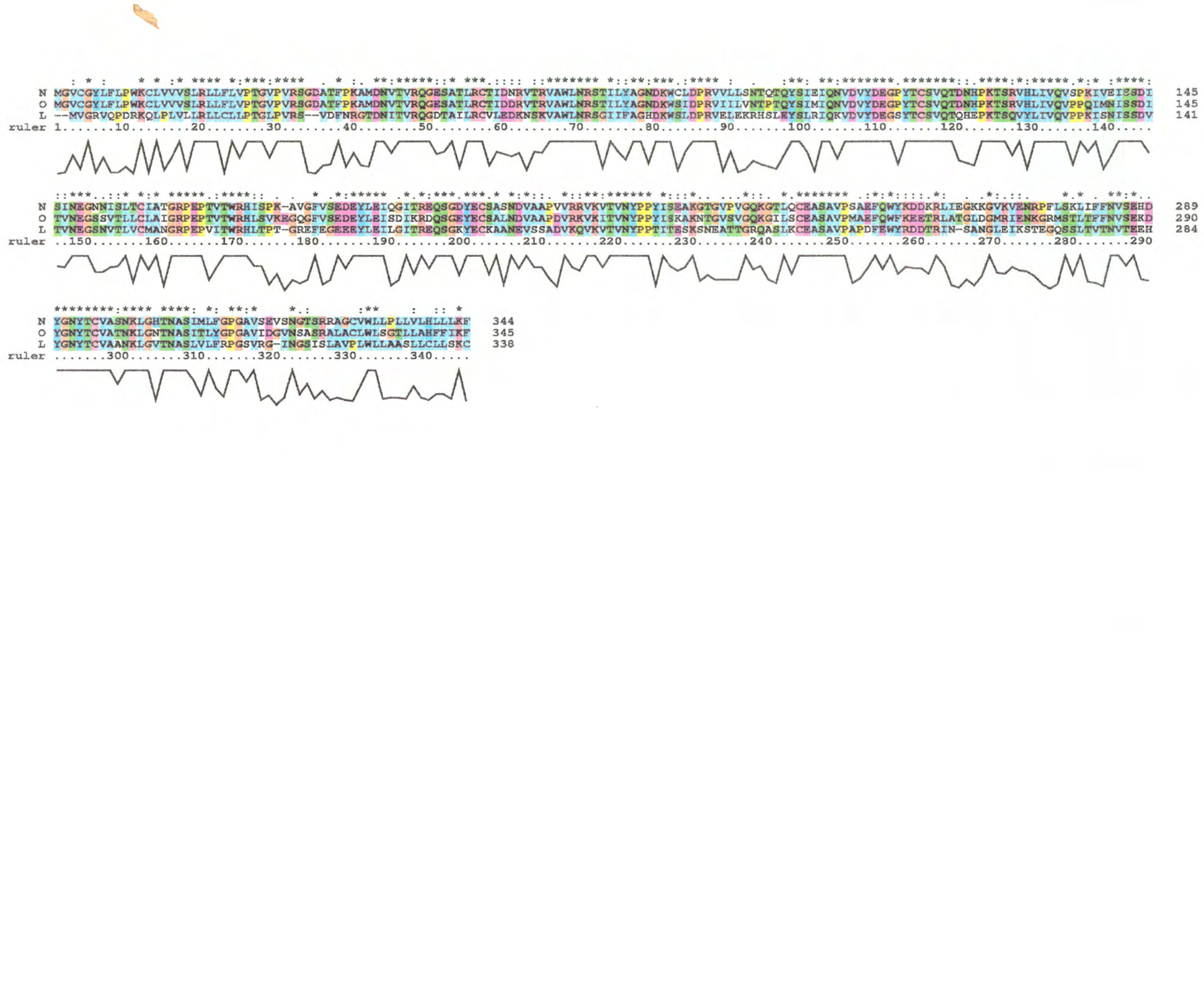
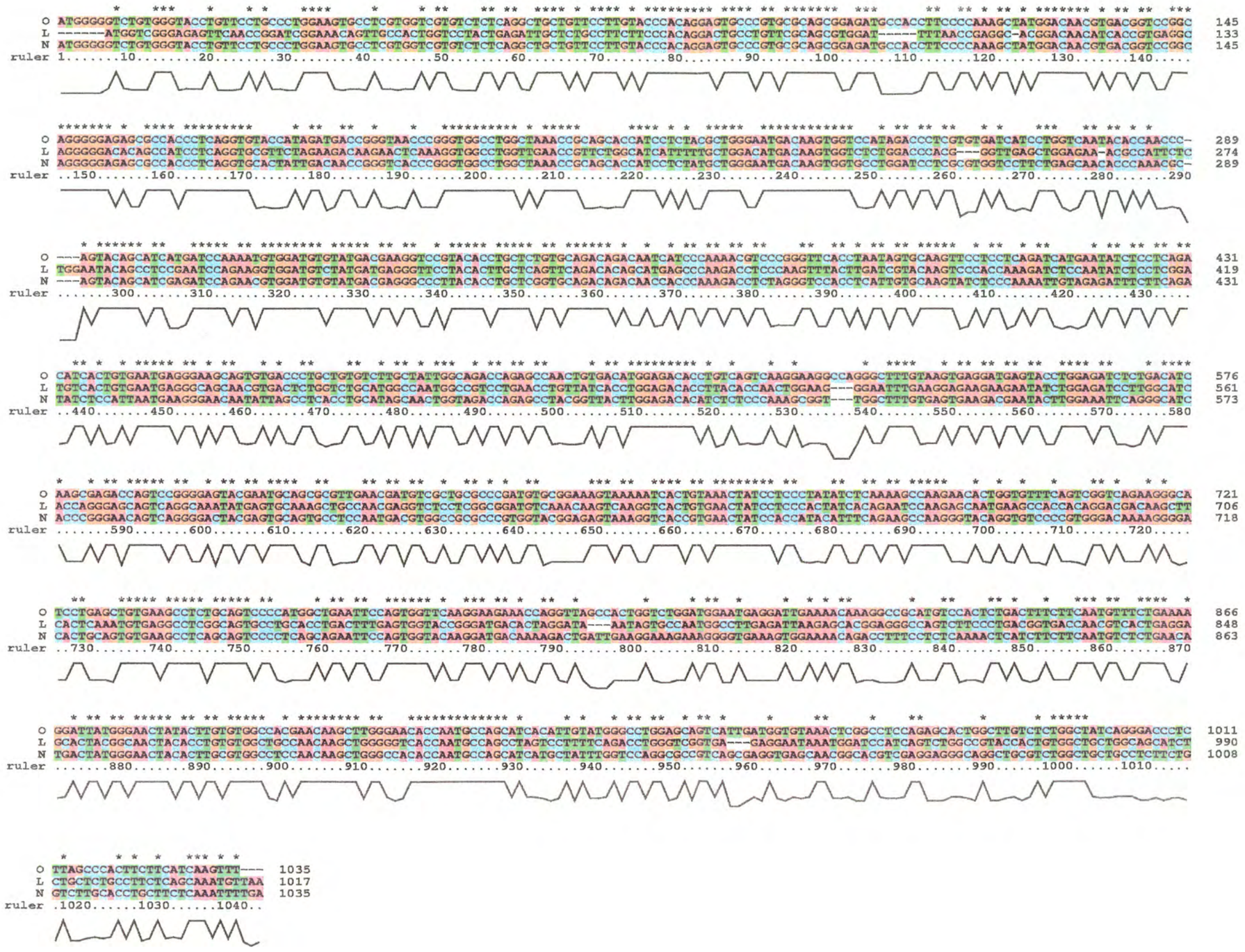


Figure 4.15 Clustalx analysis of nucleotide homology of OBCAM and the other human IgLONS



8. REFERENCES

al-Timimi, A., C. H. Buckley, and H. Fox. 1985. An immunohistochemical study of the incidence and significance of sex steroid hormone binding sites in normal and neoplastic human ovarian tissue. *Int J Gynecol Pathol* 4, no. 1: 24-41.

Alford, D. and J. Taylor-Papadimitriou. 1996. Cell adhesion molecules in the normal and cancerous mammary gland. *J Mammary Gland Biol Neoplasia* 1, no. 2: 207-18.

Alper, O., M. L. De Santis, K. Stromberg, N. F. Hacker, Y. S. Cho-Chung, and D. S. Salomon. 2000. Anti-sense suppression of epidermal growth factor receptor expression alters cellular proliferation, cell-adhesion and tumorigenicity in ovarian cancer cells. *Int J Cancer* 88, no. 4: 566-74.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J Mol Biol* 215, no. 3: 403-10.

Ando, M., S. Kol, E. Kokia, K. Ruutiainen-Altman, J. Sirois, R. M. Rohan, D. W. Payne, and E. Y. Adashi. 1998. Rat ovarian prostaglandin endoperoxide synthase-1 and -2: periovulatory expression of granulosa cell-based interleukin-1-dependent enzymes. *Endocrinology* 139, no. 5: 2501-8.

Auersperg, N., J. Pan, B. D. Grove, T. Peterson, J. Fisher, S. Maines-Bandiera, A. Somasiri, and C. D. Roskelley. 1999. E-cadherin induces mesenchymal-to-epithelial transition in human ovarian surface epithelium. *Proc Natl Acad Sci U S A* 96, no. 11: 6249-54.

Auersperg, N., A. S. Wong, K. C. Choi, S. K. Kang, and P. C. Leung. 2001. Ovarian surface epithelium: biology, endocrinology, and pathology. *Endocr Rev* 22, no. 2: 255-88.

Bahar, A. Y., P. J. Taylor, L. Andrews, A. Proos, L. Burnett, K. Tucker, M. Friedlander, and M. F. Buckley. 2001. The frequency of founder mutations in the BRCA1, BRCA2, and APC genes in Australian Ashkenazi Jews: implications for the generality of U.S. population data. *Cancer* 92, no. 2: 440-5.

Baker, V. V., M. P. Borst, D. Dixon, K. D. Hatch, H. M. Shingleton, and D. Miller. 1990. c-myc amplification in ovarian cancer. *Gynecol Oncol* 38, no. 3: 340-2.

Bates, P. A., J. Luo, and M. J. Sternberg. 1992. A predicted three-dimensional structure for the carcinoembryonic antigen (CEA). *FEBS Lett* 301, no. 2: 207-14.

Baylin, S. B. and J. G. Herman. 2000. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet* 16, no. 4: 168-74.

Behrens, J. 1999. Cadherins and catenins: role in signal transduction and tumor progression. *Cancer Metastasis Rev* 18, no. 1: 15-30.

Ben David, Y., A. Chetrit, G. Hirsh-Yechezkel, E. Friedman, B. D. Beck, U. Beller, G. Ben-Baruch, A. Fishman, H. Levavi, F. Lubin, J. Menczer, B. Piura, J. P. Struewing, and B. Modan. 2002. Effect of BRCA Mutations on the Length of Survival in Epithelial Ovarian Tumors. *J Clin Oncol* 20, no. 2: 463-6.

Ben-David, Y., E. B. Giddens, K. Letwin, and A. Bernstein. 1991. Erythroleukemia induction by Friend murine leukemia virus: insertional activation of a new member of the ets gene family, Fli-1, closely linked to c-ets-1. *Genes Dev* 5, no. 6: 908-18.

Beral, V., C. Hermon, C. Kay, P. Hannaford, S. Darby, and G. Reeves. 1999. Mortality associated with oral contraceptive use: 25 year follow up of cohort of 46 000 women from Royal College of General Practitioners' oral contraception study. *Bmj* 318, no. 7176: 96-100.

Berchuck, A., A. Kamel, R. Whitaker, B. Kerns, G. Olt, R. Kinney, J. T. Soper, R. Dodge, D. L. Clarke-Pearson, P. Marks, and et al. 1990. Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer. *Cancer Res* 50, no. 13: 4087-91.

Berns, E. M., J. G. Klijn, S. C. Henzen-Logmans, C. J. Rodenburg, M. E. van der Burg, and J. A. Foekens. 1992. Receptors for hormones and growth factors and (onco)-gene amplification in human ovarian cancer. *Int J Cancer* 52, no. 2: 218-24.
Berx, G., K. F. Becker, H. Hofler, and F. van Roy. 1998. Mutations of the human E-cadherin (CDH1) gene. *Hum Mutat* 12, no. 4: 226-37.

Biskind. 1944. *Proceedings of Society of Experimental Biological Medicine* .
Biskind. 1948. *Science* .

Brandenberger, A. W., M. K. Tee, and R. B. Jaffe. 1998. Estrogen receptor alpha (ER-alpha) and beta (ER-beta) mRNAs in normal ovary, ovarian serous cystadenocarcinoma and ovarian cancer cell lines: down-regulation of ER-beta in neoplastic tissues. *J Clin Endocrinol Metab* 83, no. 3: 1025-8.

Brash, D. E., J. A. Rudolph, J. A. Simon, A. Lin, G. J. McKenna, H. P. Baden, A. J. Halperin, and J. Ponten. 1991. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc Natl Acad Sci U S A* 88, no. 22: 10124-8.

Brock, J. A., W. H. Liu, S. T. Smith, and S. R. Young. 1996. Detection of numerical chromosome anomalies in interphase cells of ovarian carcinomas using fluorescence in situ hybridization. *Genes Chromosomes Cancer* 16, no. 2: 120-9.

Brown, D. A. and J. K. Rose. 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* 68, no. 3: 533-44.

Buck, C., S. Albelda, L. Damjanovich, J. Edelman, D. T. Shih, and J. Solowska. 1990. Immunohistochemical and molecular analysis of beta 1 and beta 3 integrins. *Cell Differ Dev* 32, no. 3: 189-202.

But, I. and B. Gorisek. 2000. DNA-ploidy as an independent prognostic factor in patients with serous ovarian carcinoma. *Int J Gynaecol Obstet* 71, no. 3: 259-62.

Caduff, R. F., S. M. Svoboda-Newman, A. W. Ferguson, and T. S. Frank. 1999. Comparison of alterations of chromosome 17 in carcinoma of the ovary and of the breast. *Virchows Arch* 434, no. 6: 517-22.

Call, K. M., T. Glaser, C. Y. Ito, A. J. Buckler, J. Pelletier, D. A. Haber, E. A. Rose, A. Kral, H. Yeger, W. H. Lewis, and et al. 1990. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* 60, no. 3: 509-20.

Cameron, E. E., K. E. Bachman, S. Myohanen, J. G. Herman, and S. B. Baylin. 1999. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* 21, no. 1: 103-7.

Campbell, I. G. and T. Manolitsas. 1999. Absence of PPP2R1B gene alterations in primary ovarian cancers. *Oncogene* 18, no. 46: 6367-9.

Cannistra, S. A., C. Ottensmeier, J. Niloff, B. Orta, and J. DiCarlo. 1995. Expression and function of beta 1 and alpha v beta 3 integrins in ovarian cancer. *Gynecol Oncol* 58, no. 2: 216-25.

Carreiras, F., Y. Denoux, C. Staedel, M. Lehmann, F. Sichel, and P. Gauduchon. 1996. Expression and localization of alpha v integrins and their ligand vitronectin in normal ovarian epithelium and in ovarian carcinoma. *Gynecol Oncol* 62, no. 2: 260-7.

Casamassimi, A., A. De Luca, S. Agrawal, K. Stromberg, D. S. Salomon, and N. Normanno. 2000. EGF-related antisense oligonucleotides inhibit the proliferation of human ovarian carcinoma cells. *Ann Oncol* 11, no. 3: 319-25.

Casasnovas, J. M., J. K. Bickford, and T. A. Springer. 1998. The domain structure of ICAM-1 and the kinetics of binding to rhinovirus. *J Virol* 72, no. 7: 6244-6.

Cavallaro, U., J. Niedermeyer, M. Fuxa, and G. Christofori. 2001. N-CAM modulates tumour-cell adhesion to matrix by inducing FGF-receptor signalling. *Nat Cell Biol* 3, no. 7: 650-7.

Cawthon, R. M., R. Weiss, G. F. Xu, D. Viskochil, M. Culver, J. Stevens, M. Robertson, D. Dunn, R. Gesteland, P. O'Connell, and et al. 1990. A major segment of the

neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. *Cell* 62, no. 1: 193-201.

Chai, Y. L., J. Cui, N. Shao, E. Shyam, P. Reddy, and V. N. Rao. 1999. The second BRCT domain of BRCA1 proteins interacts with p53 and stimulates transcription from the p21WAF1/CIP1 promoter. *Oncogene* 18, no. 1: 263-8.

Chang, J., I. Fryatt, B. Ponder, C. Fisher, and M. E. Gore. 1995. A matched control study of familial epithelial ovarian cancer: patient characteristics, response to chemotherapy and outcome. *Ann Oncol* 6, no. 1: 80-2.

Chapman, M. S. and I. M. Verma. 1996. Transcriptional activation by BRCA1. *Nature* 382, no. 6593: 678-9.

Chen, Y., W. H. Lee, and H. K. Chew. 1999. Emerging roles of BRCA1 in transcriptional regulation and DNA repair. *J Cell Physiol* 181, no. 3: 385-92.

Cheng, C. W., P. E. Wu, J. C. Yu, C. S. Huang, C. T. Yue, C. W. Wu, and C. Y. Shen. 2001. Mechanisms of inactivation of E-cadherin in breast carcinoma: modification of the two-hit hypothesis of tumor suppressor gene. *Oncogene* 20, no. 29: 3814-23.

Cheng, J. Q., A. K. Godwin, A. Bellacosa, T. Taguchi, T. F. Franke, T. C. Hamilton, P. N. Tsichlis, and J. R. Testa. 1992. AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc Natl Acad Sci U S A* 89, no. 19: 9267-71.

Chothia, C. and E. Y. Jones. 1997. The molecular structure of cell adhesion molecules. *Annu Rev Biochem* 66: 823-62.

Clarke, G. A. and D. J. Moss. 1997. GP55 inhibits both cell adhesion and growth of neurons, but not non-neuronal cells, via a G-protein-coupled receptor. *Eur J Neurosci* 9, no. 2: 334-41.

Cole, S. P., K. E. Sparks, K. Fraser, D. W. Loe, C. E. Grant, G. M. Wilson, and R. G. Deeley. 1994. Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res* 54, no. 22: 5902-10.

Cramer, D. W., R. F. Liberman, L. Titus-Ernstoff, W. R. Welch, E. R. Greenberg, J. A. Baron, and B. L. Harlow. 1999. Genital talc exposure and risk of ovarian cancer. *Int J Cancer* 81, no. 3: 351-6.

Cross, S. H. and A. P. Bird. 1995. CpG islands and genes. *Curr Opin Genet Dev* 5, no. 3: 309-14.

Cuatrecasas, M., N. Erill, E. Musulen, I. Costa, X. Matias-Guiu, and J. Prat. 1998. K-ras mutations in nonmucinous ovarian epithelial tumors: a molecular analysis and clinicopathologic study of 144 patients. *Cancer* 82, no. 6: 1088-95.

Cuatrecasas, M., A. Villanueva, X. Matias-Guiu, and J. Prat. 1997. K-ras mutations in mucinous ovarian tumors: a clinicopathologic and molecular study of 95 cases. *Cancer* 79, no. 8: 1581-6.

Damjanovich, L., S. M. Albelda, S. A. Mette, and C. A. Buck. 1992. Distribution of integrin cell adhesion receptors in normal and malignant lung tissue. *Am J Respir Cell Mol Biol* 6, no. 2: 197-206.

Darai, E., J. Y. Scoazec, F. Walker-Combrouze, N. Mlika-Cabanne, G. Feldmann, P. Madelenat, and F. Potet. 1997. Expression of cadherins in benign, borderline, and malignant ovarian epithelial tumors: a clinicopathologic study of 60 cases. *Hum Pathol* 28, no. 8: 922-8.

Davis, M., A. Hitchcock, W. D. Foulkes, and I. G. Campbell. 1996. Refinement of two chromosome 11q regions of loss of heterozygosity in ovarian cancer. *Cancer Res* 56, no. 4: 741-4.

De-Vita, Vincent T, Samuel Hellman, and Steven Rosenberg. 1997. Cancer - Principles and Practice of Oncology. , no. 5th edition.

Deng, C. X. and F. Scott. 2000. Role of the tumor suppressor gene Brca1 in genetic stability and mammary gland tumor formation. *Oncogene* 19, no. 8: 1059-64.

Diebold, J., B. Suchy, G. B. Baretton, S. Blasenbreu, W. Meier, M. Schmidt, H. Rabes, and U. Lohrs. 1996. DNA ploidy and MYC DNA amplification in ovarian carcinomas. Correlation with p53 and bcl-2 expression, proliferative activity and prognosis. *Virchows Arch* 429, no. 4-5: 221-7.

Doherty, P., S. V. Ashton, S. E. Moore, and F. S. Walsh. 1991. Morphoregulatory activities of NCAM and N-cadherin can be accounted for by G protein-dependent activation of L- and N-type neuronal Ca2+ channels. *Cell* 67, no. 1: 21-33.

Doherty, P., C. H. Barton, G. Dickson, P. Seaton, L. H. Rowett, S. E. Moore, H. J. Gower, and F. S. Walsh. 1989. Neuronal process outgrowth of human sensory neurons on monolayers of cells transfected with cDNAs for five human N-CAM isoforms. *J Cell Biol* 109, no. 2: 789-98.

Donehower, L. A., L. A. Godley, C. M. Aldaz, R. Pyle, Y. P. Shi, D. Pinkel, J. Gray, A. Bradley, D. Medina, and H. E. Varmus. 1996. The role of p53 loss in genomic instability and tumor progression in a murine mammary cancer model. *Prog Clin Biol Res* 395: 1-11.

Easton, D., D. Ford, and J. Peto. 1993. Inherited susceptibility to breast cancer. *Cancer Surv* 18: 95-113.

Edmondson, R. J., J. M. Monaghan, and B. R. Davies. 2002. The human ovarian surface epithelium is an androgen responsive tissue. *Br J Cancer* 86, no. 6: 879-85.

Ellison, P. T. and C. Lager. 1986. Moderate recreational running is associated with lowered salivary progesterone profiles in women. *Am J Obstet Gynecol* 154, no. 5: 1000-3.

Enomoto, T., C. M. Weghorst, M. Inoue, O. Tanizawa, and J. M. Rice. 1991. K-ras activation occurs frequently in mucinous adenocarcinomas and rarely in other common epithelial tumors of the human ovary. *Am J Pathol* 139, no. 4: 777-85.

Espey, L. L. 1980. Ovulation as an inflammatory reaction--a hypothesis. *Biol Reprod* 22, no. 1: 73-106.

Esteller, M., M. F. Fraga, M. Guo, J. Garcia-Foncillas, I. Hedenfalk, A. K. Godwin, J. Trojan, C. Vaurs-Barriere, Y. J. Bignon, S. Ramus, J. Benitez, T. Caldes, Y. Akiyama, Y. Yuasa, V. Launonen, M. J. Canal, R. Rodriguez, G. Capella, M. A. Peinado, A. Borg, L. A. Aaltonen, B. A. Ponder, S. B. Baylin, and J. G. Herman. 2001. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. *Hum Mol Genet* 10, no. 26: 3001-7.

Esteller, M., J. M. Silva, G. Dominguez, F. Bonilla, X. Matias-Guiu, E. Lerma, E. Bussaglia, J. Prat, I. C. Harkes, E. A. Repasky, E. Gabrielson, M. Schutte, S. B. Baylin, and J. G. Herman. 2000. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors [see comments]. *J Natl Cancer Inst* 92, no. 7: 564-9.

Fallows, S., J. Price, R. J. Atkinson, P. G. Johnston, I. Hickey, and S. E. Russell. 2001. P53 mutation does not affect prognosis in ovarian epithelial malignancies. *J Pathol* 194, no. 1: 68-75.

Fang, F., G. Orend, N. Watanabe, T. Hunter, and E. Ruoslahti. 1996. Dependence of cyclin E-CDK2 kinase activity on cell anchorage. *Science* 271, no. 5248: 499-502.

Fathalla, M. F. 1971a. Incessant ovulation--a factor in ovarian neoplasia? *Lancet* 2, no. 7716: 163.

Fathalla, M. F. 1971b. Incessant ovulation--a factor in ovarian neoplasia? *Lancet* 2, no. 7716: 163.

Fazeli, A., S. L. Dickinson, M. L. Hermiston, R. V. Tighe, R. G. Steen, C. G. Small, E. T. Stoeckli, K. Keino-Masu, M. Masu, H. Rayburn, J. Simons, R. T. Bronson, J. I.

Gordon, M. Tessier-Lavigne, and R. A. Weinberg. 1997. Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. *Nature* 386, no. 6627: 796-804.

Fearon, E. R., K. R. Cho, J. M. Nigro, S. E. Kern, J. W. Simons, J. M. Ruppert, S. R. Hamilton, A. C. Preisinger, G. Thomas, K. W. Kinzler, and et al. 1990. Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science* 247, no. 4938: 49-56.

Fogar, P., D. Basso, C. Pasquali, M. De Paoli, C. Sperti, G. Roveroni, S. Pedrazzoli, and M. Plebani. 1997. Neural cell adhesion molecule (N-CAM) in gastrointestinal neoplasias. *Anticancer Res* 17, no. 2B: 1227-30.

Folkman, J. and A. Moscona. 1978. Role of cell shape in growth control. *Nature* 273, no. 5661: 345-9.

Ford, D., D. F. Easton, M. Stratton, S. Narod, D. Goldgar, P. Devilee, D. T. Bishop, B. Weber, G. Lenoir, J. Chang-Claude, H. Sobol, M. D. Teare, J. Struewing, A. Arason, S. Scherneck, J. Peto, T. R. Rebbeck, P. Tonin, S. Neuhausen, R. Barkardottir, J. Eyfjord, H. Lynch, B. A. Ponder, S. A. Gayther, M. Zelada-Hedman, and et al. 1998. Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. *Am J Hum Genet* 62, no. 3: 676-89.

Foulkes, W. D., I. G. Campbell, G. W. Stamp, and J. Trowsdale. 1993. Loss of heterozygosity and amplification on chromosome 11q in human ovarian cancer. *Br J Cancer* 67, no. 2: 268-73.

Friedrichson, T. and T. V. Kurzchalia. 1998. Microdomains of GPI-anchored proteins in living cells revealed by crosslinking. *Nature* 394, no. 6695: 802-5.

Friend, S. H., J. M. Horowitz, M. R. Gerber, X. F. Wang, E. Bogenmann, F. P. Li, and R. A. Weinberg. 1987. Deletions of a DNA sequence in retinoblastomas and mesenchymal tumors: organization of the sequence and its encoded protein. *Proc Natl Acad Sci U S A* 84, no. 24: 9059-63.

Frisch, S. M. and H. Francis. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* 124, no. 4: 619-26.

Funatsu, N., S. Miyata, H. Kumanogoh, M. Shigeta, K. Hamada, Y. Endo, Y. Sokawa, and S. Maekawa. 1999. Characterization of a novel rat brain glycosylphosphatidylinositol-anchored protein (Kilon), a member of the IgLON cell adhesion molecule family. *J Biol Chem* 274, no. 12: 8224-30.

Gabra, H., L. Taylor, B. B. Cohen, A. Lessels, D. M. Eccles, R. C. Leonard, J. F. Smyth, and C. M. Steel. 1995. Chromosome 11 allele imbalance and clinicopathological correlates in ovarian tumours. *Br J Cancer* 72, no. 2: 367-75.

Gabra, H., J. E. Watson, K. J. Taylor, J. Mackay, R. C. Leonard, C. M. Steel, D. J. Porteous, and J. F. Smyth. 1996. Definition and refinement of a region of loss of heterozygosity at 11q23.3-q24.3 in epithelial ovarian cancer associated with poor prognosis. *Cancer Res* 56, no. 5: 950-4.

Gayther, S. A., J. Mangion, P. Russell, S. Seal, R. Barfoot, B. A. Ponder, M. R. Stratton, and D. Easton. 1997. Variation of risks of breast and ovarian cancer associated with different germline mutations of the BRCA2 gene. *Nat Genet* 15, no. 1: 103-5.

Gessler, M., A. Poustka, W. Cavenee, R. L. Neve, S. H. Orkin, and G. A. Bruns. 1990. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. *Nature* 343, no. 6260: 774-8.

Gil, O. D., G. Zanazzi, A. F. Struyk, and J. L. Salzer. 1998. Neurotrimin mediates bifunctional effects on neurite outgrowth via homophilic and heterophilic interactions. *J Neurosci* 18, no. 22: 9312-25.

Graff, J. R., E. Gabrielson, H. Fujii, S. B. Baylin, and J. G. Herman. 2000. Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. *J Biol Chem* 275, no. 4: 2727-32.

Graff, J. R., V. E. Greenberg, J. G. Herman, W. H. Westra, E. R. Boghaert, K. B. Ain, M. Saji, M. A. Zeiger, S. G. Zimmer, and S. B. Baylin. 1998. Distinct patterns of E-cadherin CpG island methylation in papillary, follicular, Hurthle's cell, and poorly differentiated human thyroid carcinoma. *Cancer Res* 58, no. 10: 2063-6.

Gras, E., J. Cortes, O. Diez, C. Alonso, X. Matias-Guiu, M. Baiget, and J. Prat. 2001. Loss of heterozygosity on chromosome 13q12-q14, BRCA-2 mutations and lack of BRCA-2 promoter hypermethylation in sporadic epithelial ovarian tumors. *Cancer* 92, no. 4: 787-95.

Green, A., D. Purdie, C. Bain, V. Siskind, P. Russell, M. Quinn, and B. Ward. 1997. Tubal sterilisation, hysterectomy and decreased risk of ovarian cancer. Survey of Women's Health Study Group. *Int J Cancer* 71, no. 6: 948-51.

Guadagno, T. M., M. Ohtsubo, J. M. Roberts, and R. K. Assoian. 1993. A link between cyclin A expression and adhesion-dependent cell cycle progression. *Science* 262, no. 5139: 1572-5.

Gumbiner, B. M. 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 84, no. 3: 345-57.

Haber, D. and E. Harlow. 1997. Tumour-suppressor genes: evolving definitions in the genomic age. *Nat Genet* 16, no. 4: 320-2.

Hachisuka, A., T. Yamazaki, J. Sawada, and T. Terao. 1996. Characterization and tissue distribution of opioid-binding cell adhesion molecule (OBCAM) using monoclonal antibodies. *Neurochem Int* 28, no. 4: 373-9.

Hahm, K. B., K. Cho, C. Lee, Y. H. Im, J. Chang, S. G. Choi, P. H. Sorensen, C. J. Thiele, and S. J. Kim. 1999. Repression of the gene encoding the TGF-beta type II receptor is a major target of the EWS-FLI1 oncoprotein. *Nat Genet* 23, no. 2: 222-7.

Hampton, G. M., C. Howe, G. Leuteritz, H. Thomas, W. F. Bodmer, E. Solomon, and W. G. Ballhausen. 1991. Regional mapping of 22 microclones around the adenomatous polyposis coli (APC) locus on chromosome 5q. *Hum Genet* 88, no. 1: 112-4.

Harpaz, Y. and C. Chothia. 1994. Many of the immunoglobulin superfamily domains in cell adhesion molecules and surface receptors belong to a new structural set which is close to that containing variable domains. *J Mol Biol* 238, no. 4: 528-39.

Hashida, H., A. Takabayashi, M. Adachi, T. Imai, K. Kondo, N. Kohno, Y. Yamaoka, and M. Miyake. 2001. The novel monoclonal antibody MH8-4 inhibiting cell motility recognizes integrin alpha3: inverse of its expression with metastases in colon cancer [In Process Citation]. *Int J Oncol* 18, no. 1: 89-95.

Hedrick, L., K. R. Cho, E. R. Fearon, T. C. Wu, K. W. Kinzler, and B. Vogelstein. 1994. The DCC gene product in cellular differentiation and colorectal tumorigenesis. *Genes Dev* 8, no. 10: 1174-83.

Helzlsouer, K. J., A. J. Alberg, G. B. Gordon, C. Longcope, T. L. Bush, S. C. Hoffman, and G. W. Comstock. 1995. Serum gonadotropins and steroid hormones and the development of ovarian cancer. *Jama* 274, no. 24: 1926-30.

Herman, J. G., F. Latif, Y. Weng, M. I. Lerman, B. Zbar, S. Liu, D. Samid, D. S. Duan, J. R. Gnarra, W. M. Linehan, and et al. 1994. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci U S A* 91, no. 21: 9700-4.

Herman, J. G., A. Umar, K. Polyak, J. R. Graff, N. Ahuja, J. P. Issa, S. Markowitz, J. K. Willson, S. R. Hamilton, K. W. Kinzler, M. F. Kane, R. D. Kolodner, B. Vogelstein, T. A. Kunkel, and S. B. Baylin. 1998. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci U S A* 95, no. 12: 6870-5.

Higgins, J. M., D. A. Mandlebrot, S. K. Shaw, G. J. Russell, E. A. Murphy, Y. T. Chen, W. J. Nelson, C. M. Parker, and M. B. Brenner. 1998. Direct and regulated interaction of integrin alphaEbeta7 with E-cadherin. *J Cell Biol* 140, no. 1: 197-210.

Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris. 1991. p53 mutations in human cancers. *Science* 253, no. 5015: 49-53.

Hooper, M. L. 1998. Tumour suppressor gene mutations in humans and mice: parallels and contrasts. *Embo J* 17, no. 23: 6783-9.

Hynes, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69, no. 1: 11-25.

Hynes, R. O. 1999. Cell adhesion: old and new questions. *Trends Cell Biol* 9, no. 12: M33-7.

Inoue, M., H. Ogawa, M. Miyata, H. Shiozaki, and O. Tanizawa. 1992. Expression of E-cadherin in normal, benign, and malignant tissues of female genital organs. *Am J Clin Pathol* 98, no. 1: 76-80.

Ivarsson, K., K. Sundfeldt, M. Brannstrom, and P. O. Janson. 2001. Production of steroids by human ovarian surface epithelial cells in culture: possible role of progesterone as growth inhibitor. *Gynecol Oncol* 82, no. 1: 116-21.

Kallakury, B. V., C. E. Sheehan, E. Winn-Deen, J. Oliver, H. A. Fisher, R. P. Kaufman, Jr., and J. S. Ross. 2001. Decreased expression of catenins (alpha and beta), p120 CTN, and E-cadherin cell adhesion proteins and E-cadherin gene promoter methylation in prostatic adenocarcinomas. *Cancer* 92, no. 11: 2786-95.

Kato, I., S. Tominaga, and C. Terao. 1989. Alcohol consumption and cancers of hormone-related organs in females. *Jpn J Clin Oncol* 19, no. 3: 202-7.

Kaufman, D. W., J. P. Kelly, W. R. Welch, L. Rosenberg, P. D. Stolley, M. E. Warshauer, J. Lewis, J. Woodruff, and S. Shapiro. 1989. Noncontraceptive estrogen use and epithelial ovarian cancer. *Am J Epidemiol* 130, no. 6: 1142-51.

Kaunitz, A. M. 1996. Depot medroxyprogesterone acetate contraception and the risk of breast and gynecologic cancer. *J Reprod Med* 41, no. 5 Suppl: 419-27.

Kawakami, M., J. Staub, W. Cliby, L. Hartmann, D. I. Smith, and V. Shridhar. 1999. Involvement of H-cadherin (CDH13) on 16q in the region of frequent deletion in ovarian cancer. *Int J Oncol* 15, no. 4: 715-20.

Kiechle-Schwarz, M., T. Bauknecht, J. Schmidt, L. Walz, and A. Pfleiderer. 1995. Recurrent cytogenetic aberrations in human ovarian carcinomas. *Cancer Detect Prev* 19, no. 3: 234-43.

Kimmig, R., P. Wimberger, P. Hillemanns, T. Kapsner, C. Caspari, and H. Hepp. 2002. Multivariate analysis of the prognostic significance of DNA-ploidy and S-phase fraction

in ovarian cancer determined by flow cytometry following detection of cytokeratin-labeled tumor cells. *Gynecol Oncol* 84, no. 1: 21-31.

Knudson, A. G., Jr. 1971. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 68, no. 4: 820-3.

Kohler, M., I. Janz, H. O. Wintzer, E. Wagner, and T. Bauknecht. 1989. The expression of EGF receptors, EGF-like factors and c-myc in ovarian and cervical carcinomas and their potential clinical significance. *Anticancer Res* 9, no. 6: 1537-47.

Koike, M., S. Takeuchi, S. Park, Y. Hatta, J. Yokota, N. Tsuruoka, and H. P. Koeffler. 1999. Ovarian cancer: loss of heterozygosity frequently occurs in the ATM gene, but structural alterations do not occur in this gene. *Oncology* 56, no. 2: 160-3.

Kol, S., I. Kehat, and E. Y. Adashi. 2002. Ovarian interleukin-1-induced gene expression: privileged genes threshold theory. *Med Hypotheses* 58, no. 1: 6-8.

Kuper, H., L. Titus-Ernstoff, B. L. Harlow, and D. W. Cramer. 2000. Population based study of coffee, alcohol and tobacco use and risk of ovarian cancer. *Int J Cancer* 88, no. 2: 313-8.

Kushi, L. H., P. J. Mink, A. R. Folsom, K. E. Anderson, W. Zheng, D. Lazovich, and T. A. Sellers. 1999. Prospective study of diet and ovarian cancer. *Am J Epidemiol* 149, no. 1: 21-31.

La Vecchia, C., E. Negri, S. Franceschi, F. Parazzini, A. Gentile, and M. Fasoli. 1992. Alcohol and epithelial ovarian cancer. *J Clin Epidemiol* 45, no. 9: 1025-30.

Lander, E. S. 2001. Initial sequencing and analysis of the human genome. *Nature* 409, no. 6822: 860-921.

Larue, L., M. Ohsugi, J. Hirchenhain, and R. Kemler. 1994. E-cadherin null mutant embryos fail to form a trophectoderm epithelium. *Proc Natl Acad Sci U S A* 91, no. 17: 8263-7.

Latif, F., K. Tory, J. Gnarra, M. Yao, F. M. Duh, M. L. Orcutt, T. Stackhouse, I. Kuzmin, W. Modi, L. Geil, and et al. 1993. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 260, no. 5112: 1317-20.

Launonen, V., F. Stenback, U. Puistola, R. Bloigu, P. Huusko, S. Kytola, A. Kauppila, and R. Winqvist. 1998. Chromosome 11q22.3-q25 LOH in ovarian cancer: association with a more aggressive disease course and involved subregions. *Gynecol Oncol* 71, no. 2: 299-304.

Lerman, M. I. and J. D. Minna. 2000. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. *Cancer Res* 60, no. 21: 6116-33.

Lessey, B. A., S. Albelda, C. A. Buck, A. J. Castelbaum, I. Yeh, M. Kohler, and A. Berchuck. 1995. Distribution of integrin cell adhesion molecules in endometrial cancer. *Am J Pathol* 146, no. 3: 717-26.

Levitt, P. 1984. A monoclonal antibody to limbic system neurons. *Science* 223, no. 4633: 299-301.

Lewis, J. D., R. R. Meehan, W. J. Henzel, I. Maurer-Fogy, P. Jeppesen, F. Klein, and A. Bird. 1992. Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. *Cell* 69, no. 6: 905-14.

Lodge, A. P., M. R. Howard, C. J. McNamee, and D. J. Moss. 2000. Co-localisation, heterophilic interactions and regulated expression of IgLON family proteins in the chick nervous system. *Brain Res Mol Brain Res* 82, no. 1-2: 84-94.

Lynch, H. T., W. Kimberling, W. A. Albano, J. F. Lynch, K. Biscone, G. S. Schuelke, A. A. Sandberg, M. Lipkin, E. E. Deschner, Y. B. Mikol, and et al. 1985a. Hereditary nonpolyposis colorectal cancer (Lynch syndromes I and II). I. Clinical description of resource. *Cancer* 56, no. 4: 934-8.

Lynch, H. T., G. S. Schuelke, W. J. Kimberling, W. A. Albano, J. F. Lynch, K. A. Biscone, M. L. Lipkin, E. E. Deschner, Y. B. Mikol, A. A. Sandberg, and et al. 1985b. Hereditary nonpolyposis colorectal cancer (Lynch syndromes I and II). II. Biomarker studies. *Cancer* 56, no. 4: 939-51.

Machado, J. C., C. Oliveira, R. Carvalho, P. Soares, G. Berx, C. Caldas, R. Seruca, F. Carneiro, and M. Sobrinho-Simoes. 2001. E-cadherin gene (CDH1) promoter methylation as the second hit in sporadic diffuse gastric carcinoma. *Oncogene* 20, no. 12: 1525-8.

Marchbanks, P. A., H. Wilson, E. Bastos, D. W. Cramer, J. M. Schildkraut, and H. B. Peterson. 2000. Cigarette smoking and epithelial ovarian cancer by histologic type. *Obstet Gynecol* 95, no. 2: 255-60.

Marg, A., P. Sirim, F. Spaltmann, A. Plagge, G. Kauselmann, F. Buck, F. G. Rathjen, and T. Brummendorf. 1999. Neurotractin, a novel neurite outgrowth-promoting Ig-like protein that interacts with CEPU-1 and LAMP. *J Cell Biol* 145, no. 4: 865-76.

Masuda, H., T. Tanaka, H. Matsuda, and I. Kusaba. 1990. Increased removal of DNA-bound platinum in a human ovarian cancer cell line resistant to cis-diamminedichloroplatinum(II). *Cancer Res* 50, no. 6: 1863-6.

Matsumura, T., R. Makino, and K. Mitamura. 2001. Frequent down-regulation of E-cadherin by genetic and epigenetic changes in the malignant progression of hepatocellular carcinomas. *Clin Cancer Res* 7, no. 3: 594-9.

Matsuoka, T., M. Yashiro, S. Nishimura, T. Inoue, T. Fujihara, T. Sawada, Y. Kato, S. Seki, and K. Hirakawa-Ys Chung. 2000. Increased expression of alpha2beta1-integrin in the peritoneal dissemination of human gastric carcinoma. *Int J Mol Med* 5, no. 1: 21-5.
Mavrothalassitis, G. and J. Ghysdael. 2000. Proteins of the ETS family with transcriptional repressor activity. *Oncogene* 19, no. 55: 6524-32.

McNamee, C. J., J. E. Reed, M. R. Howard, A. P. Lodge, and D. J. Moss. 2002. Promotion of neuronal cell adhesion by members of the IgLON family occurs in the absence of either support or modification of neurite outgrowth. *J Neurochem* 80, no. 6: 941-8.

McNatty, K. P., A. Makris, C. De Grazia, R. Osathanondh, and K. J. Ryan. 1979. The production of progesterone, androgens and oestrogens by human granulosa cells in vitro and in vivo. *J Steroid Biochem* 11, no. 1C: 775-9.

Meden, H. and W. Kuhn. 1997. Overexpression of the oncogene c-erbB-2 (HER2/neu) in ovarian cancer: a new prognostic factor. *Eur J Obstet Gynecol Reprod Biol* 71, no. 2: 173-9.

Meredith, J. E., Jr., B. Fazeli, and M. A. Schwartz. 1993. The extracellular matrix as a cell survival factor. *Mol Biol Cell* 4, no. 9: 953-61.

Merlo, A., J. G. Herman, L. Mao, D. J. Lee, E. Gabrielson, P. C. Burger, S. B. Baylin, and D. Sidransky. 1995. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat Med* 1, no. 7: 686-92.

Miki, Y., J. Swensen, D. Shattuck-Eidens, P. A. Futreal, K. Harshman, S. Tavtigian, Q. Liu, C. Cochran, L. M. Bennett, W. Ding, and et al. 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 266, no. 5182: 66-71.

Mink, P. J., A. R. Folsom, T. A. Sellers, and L. H. Kushi. 1996. Physical activity, waist-to-hip ratio, and other risk factors for ovarian cancer: a follow-up study of older women. *Epidemiology* 7, no. 1: 38-45.

Modan, B., P. Hartge, G. Hirsh-Yechezkel, A. Chetrit, F. Lubin, U. Beller, G. Ben-Baruch, A. Fishman, J. Menczer, S. M. Ebbers, M. A. Tucker, S. Wacholder, J. P.

Struewing, E. Friedman, and B. Piura. 2001. Parity, oral contraceptives, and the risk of ovarian cancer among carriers and noncarriers of a BRCA1 or BRCA2 mutation. *N Engl J Med* 345, no. 4: 235-40.

Muller, A., B. Homey, H. Soto, N. Ge, D. Catron, M. E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S. N. Wagner, J. L. Barrera, A. Mohar, E. Verastegui, and A. Zlotnik. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410, no. 6824: 50-6.

Nacheva, E. P., C. D. Grace, M. Bittner, D. H. Ledbetter, R. B. Jenkins, and A. R. Green. 1998. Comparative genomic hybridization: a comparison with molecular and cytogenetic analysis. *Cancer Genet Cytogenet* 100, no. 2: 93-105.

Nagai, M. A., L. Yamamoto, S. Salaorni, M. M. Pacheco, M. M. Brentani, E. M. Barbosa, R. R. Brentani, S. Mazoyer, S. A. Smith, B. A. Ponder, and et al. 1994. Detailed deletion mapping of chromosome segment 17q12-21 in sporadic breast tumours. *Genes Chromosomes Cancer* 11, no. 1: 58-62.

Nagai, N., T. Oshita, T. Fujii, Y. Katsube, S. Matsubayashi, and K. Ohama. 2001. Are DNA ploidy and epidermal growth factor receptor prognostic factors for untreated ovarian cancer? A prospective study. *Am J Clin Oncol* 24, no. 3: 215-21.

Nakayama, S., A. Sasaki, H. Mese, R. E. Alcalde, T. Tsuji, and T. Matsumura. 2001. The E-cadherin gene is silenced by CpG methylation in human oral squamous cell carcinomas. *Int J Cancer* 93, no. 5: 667-73.

Negrini, M., S. Sabbioni, S. Haldar, L. Possati, A. Castagnoli, A. Corallini, G. Barbanti-Brodano, and C. M. Croce. 1994. Tumor and growth suppression of breast cancer cells by chromosome 17-associated functions. *Cancer Res* 54, no. 7: 1818-24.

Ness, R. B. and C. Cottreau. 1999. Possible role of ovarian epithelial inflammation in ovarian cancer. *J Natl Cancer Inst* 91, no. 17: 1459-67.

Nicholson, K. M. and N. G. Anderson. 2002. The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* 14, no. 5: 381-95.

Nojima, D., K. Nakajima, L. C. Li, J. Franks, L. Ribeiro-Filho, N. Ishii, and R. Dahiya. 2001. CpG methylation of promoter region inactivates E-cadherin gene in renal cell carcinoma. *Mol Carcinog* 32, no. 1: 19-27.

Oda, K., H. Arakawa, T. Tanaka, K. Matsuda, C. Tanikawa, T. Mori, H. Nishimori, K. Tamai, T. Tokino, Y. Nakamura, and Y. Taya. 2000. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* 102, no. 6: 849-62.

Okamoto, A., Y. Sameshima, S. Yokoyama, Y. Terashima, T. Sugimura, M. Terada, and J. Yokota. 1991. Frequent allelic losses and mutations of the p53 gene in human ovarian cancer. *Cancer Res* 51, no. 19: 5171-6.

Ong, A., S. L. Maines-Bandiera, C. D. Roskelley, and N. Auersperg. 2000. An ovarian adenocarcinoma line derived from SV40/E-cadherin-transfected normal human ovarian surface epithelium. *Int J Cancer* 85, no. 3: 430-7.

O'Toole TE, Katagiri Y, Faull RJ, Peter K, Tamura R, Quaranta V, Loftus JC, Shattil SJ, Ginsberg MH 1994. Integrin cytoplasmic domains mediate inside-out signal transduction. *J Cell Biol.* Mar; no.124(6):1047-59.

Ozaki, I., K. Yamamoto, T. Mizuta, S. Kajihara, N. Fukushima, Y. Setoguchi, F. Morito, and T. Sakai. 1998. Differential expression of laminin receptors in human hepatocellular carcinoma. *Gut* 43, no. 6: 837-42.

Ozalp, S., O. T. Yalcin, Z. Gulbas, H. M. Tanir, and T. Minsin. 2001. Effect of cellular DNA content on the prognosis of epithelial ovarian cancers. *Gynecol Obstet Invest* 52, no. 2: 93-7.

Pejovic, T. 1995. Genetic changes in ovarian cancer. *Ann Med* 27, no. 1: 73-8.

Peralta Soler, A., K. A. Knudsen, A. Tecson-Miguel, F. X. McBrearty, A. C. Han, and H. Salazar. 1997. Expression of E-cadherin and N-cadherin in surface epithelial-stromal tumors of the ovary distinguishes mucinous from serous and endometrioid tumors. *Hum Pathol* 28, no. 6: 734-9

.

Perl, A. K., U. Dahl, P. Wilgenbus, H. Cremer, H. Semb, and G. Christofori. 1999. Reduced expression of neural cell adhesion molecule induces metastatic dissemination of pancreatic beta tumor cells. *Nat Med* 5, no. 3: 286-91.

Perl, A. K., P. Wilgenbus, U. Dahl, H. Semb, and G. Christofori. 1998. A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* 392, no. 6672: 190-3.

Perlino, E., M. Lovecchio, R. A. Vacca, M. Fornaro, L. Moro, P. Ditonno, M. Battaglia, F. P. Selvaggi, M. G. Mastropasqua, P. Bufo, and L. R. Languino. 2000. Regulation of mRNA and protein levels of beta1 integrin variants in human prostate carcinoma [In Process Citation]. *Am J Pathol* 157, no. 5: 1727-34.

Pimenta, A. F., I. Fischer, and P. Levitt. 1996. cDNA cloning and structural analysis of the human limbic-system-associated membrane protein (LAMP). *Gene* 170, no. 2: 189-95.

Polychronopoulou, A., A. Tzonou, C. C. Hsieh, G. Kaprinis, A. Rebelakos, N. Toupadaki, and D. Trichopoulos. 1993. Reproductive variables, tobacco, ethanol, coffee and somatometry as risk factors for ovarian cancer. *Int J Cancer* 55, no. 3: 402-7.

Purdie, D., A. Green, C. Bain, V. Siskind, B. Ward, N. Hacker, M. Quinn, G. Wright, P. Russell, and B. Susil. 1995a. Reproductive and other factors and risk of epithelial ovarian cancer: an Australian case-control study. Survey of Women's Health Study Group. *Int J Cancer* 62, no. 6: 678-84.

Purdie, D., A. Green, C. Bain, V. Siskind, B. Ward, N. Hacker, M. Quinn, G. Wright, P. Russell, and B. Susil. 1995b. Reproductive and other factors and risk of epithelial ovarian cancer: an Australian case-control study. Survey of Women's Health Study Group. *Int J Cancer* 62, no. 6: 678-84.

Rai, S. K., F. M. Duh, V. Vigdorovich, A. Danilkovitch-Miagkova, M. I. Lerman, and A. D. Miller. 2001. Candidate tumor suppressor HYAL2 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor for jaagsiekte sheep retrovirus, the envelope protein of which mediates oncogenic transformation. *Proc Natl Acad Sci U S A* 98, no. 8: 4443-8.

Rao, P. H., C. P. Harris, X. Yan Lu, X. N. Li, S. C. Mok, and C. C. Lau. 2002. Multicolor spectral karyotyping of serous ovarian adenocarcinoma. *Genes Chromosomes Cancer* 33, no. 2: 123-32.

Reale, M. A., G. Hu, A. I. Zafar, R. H. Getzenberg, S. M. Levine, and E. R. Fearon. 1994. Expression and alternative splicing of the deleted in colorectal cancer (DCC) gene in normal and malignant tissues. *Cancer Res* 54, no. 16: 4493-501.

Riethmacher, D., V. Brinkmann, and C. Birchmeier. 1995. A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. *Proc Natl Acad Sci U S A* 92, no. 3: 855-9.

Riman, T., P. W. Dickman, S. Nilsson, N. Correia, H. Nordlinder, C. M. Magnusson, E. Weiderpass, and I. R. Persson. 2002. Hormone replacement therapy and the risk of invasive epithelial ovarian cancer in Swedish women. *J Natl Cancer Inst* 94, no. 7: 497-504.

Risch, H. A. 1996. Estrogen replacement therapy and risk of epithelial ovarian cancer. *Gynecol Oncol* 63, no. 2: 254-7.

Risch, H. A. 1997. Re: Relationship between lifetime ovulatory cycles and overexpression of mutant p53 in epithelial ovarian cancer. *J Natl Cancer Inst* 89, no. 22: 1726-7.

Risch, H. A., M. Jain, L. D. Marrett, and G. R. Howe. 1994. Dietary fat intake and risk of epithelial ovarian cancer. *J Natl Cancer Inst* 86, no. 18: 1409-15.

Risch, H. A., L. D. Marrett, and G. R. Howe. 1994. Parity, contraception, infertility, and the risk of epithelial ovarian cancer. *Am J Epidemiol* 140, no. 7: 585-97.

Risinger, J. I., A. Berchuck, M. F. Kohler, and J. Boyd. 1994. Mutations of the E-cadherin gene in human gynecologic cancers. *Nat Genet* 7, no. 1: 98-102.

Robertson, K. D., E. Uzvolgyi, G. Liang, C. Talmadge, J. Sumegi, F. A. Gonzales, and P. A. Jones. 1999. The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res* 27, no. 11: 2291-8.

Rouleau, G. A., P. Merel, M. Lutchman, M. Sanson, J. Zucman, C. Marineau, K. Hoang-Xuan, S. Demczuk, C. Desmaze, B. Plougastel, and et al. 1993. Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. *Nature* 363, no. 6429: 515-21.

Sadler, T.W. 1990. Langman's Medical Embryology. Sixth Edition.

Saegusa, M., D. Machida, and I. Okayasu. 2000. Loss of DCC gene expression during ovarian tumorigenesis: relation to tumour differentiation and progression. *Br J Cancer* 82, no. 3: 571-8.

Sato, S., J. Kigawa, Y. Minagawa, M. Okada, M. Shimada, M. Takahashi, S. Kamazawa, and N. Terakawa. 1999. Chemosensitivity and p53-dependent apoptosis in epithelial ovarian carcinoma. *Cancer* 86, no. 7: 1307-13.

Schildkraut, J. M., P. J. Schwingl, E. Bastos, A. Evanoff, and C. Hughes. 1996. Epithelial ovarian cancer risk among women with polycystic ovary syndrome. *Obstet Gynecol* 88, no. 4 Pt 1: 554-9.

Schofield, P. R., K. C. McFarland, J. S. Hayflick, J. N. Wilcox, T. M. Cho, S. Roy, N. M. Lee, H. H. Loh, and P. H. Seeburg. 1989. Molecular characterization of a new immunoglobulin superfamily protein with potential roles in opioid binding and cell contact. *Embo J* 8, no. 2: 489-95.

Scully, R., S. Ganesan, K. Vlasakova, J. Chen, M. Socolovsky, and D. M. Livingston. 1999. Genetic analysis of BRCA1 function in a defined tumor cell line. *Mol Cell* 4, no. 6: 1093-9.

Sellar, G. C., L. Li, K. P. Watt, B. D. Nelkin, G. J. Rabiasz, E. A. Stronach, E. P. Miller, D. J. Porteous, J. F. Smyth, and H. Gabra. 2001. BARX2 induces cadherin 6 expression and is a functional suppressor of ovarian cancer progression. *Cancer Res* 61, no. 19: 6977-81.

Shahin, M. S., J. H. Hughes, A. K. Sood, and R. E. Buller. 2000. The prognostic significance of p53 tumor suppressor gene alterations in ovarian carcinoma. *Cancer* 89, no. 9: 2006-17.

Shapiro, L., A. M. Fannon, P. D. Kwong, A. Thompson, M. S. Lehmann, G. Grubel, J. F. Legrand, J. Als-Nielsen, D. R. Colman, and W. A. Hendrickson. 1995. Structural basis of cell-cell adhesion by cadherins. *Nature* 374, no. 6520: 327-37.

Shark, K. B. and N. M. Lee. 1995. Cloning, sequencing and localization to chromosome 11 of a cDNA encoding a human opioid-binding cell adhesion molecule (OBCAM). *Gene* 155, no. 2: 213-7.

Shaw, L. M. 1999. Integrin function in breast carcinoma progression. *J Mammary Gland Biol Neoplasia* 4, no. 4: 367-76.

Shen, S. X., Z. Weaver, X. Xu, C. Li, M. Weinstein, L. Chen, X. Y. Guan, T. Ried, and C. X. Deng. 1998. A targeted disruption of the murine Brca1 gene causes gamma-irradiation hypersensitivity and genetic instability. *Oncogene* 17, no. 24: 3115-24.

Si, H. X., S. W. Tsao, K. Y. Lam, G. Srivastava, Y. Liu, Y. C. Wong, Z. Y. Shen, and A. L. Cheung. 2001. E-cadherin expression is commonly downregulated by CpG island hypermethylation in esophageal carcinoma cells. *Cancer Lett* 173, no. 1: 71-8.

Siskind, V., A. Green, C. Bain, and D. Purdie. 1997. Breastfeeding, menopause, and epithelial ovarian cancer. *Epidemiology* 8, no. 2: 188-91.

Springer, T. A. 1990. The sensation and regulation of interactions with the extracellular environment: the cell biology of lymphocyte adhesion receptors. *Annu Rev Cell Biol* 6: 359-402.

Strathdee, G., M. J. MacKean, M. Illand, and R. Brown. 1999. A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer. *Oncogene* 18, no. 14: 2335-41.

Struyk, A. F., P. D. Canoll, M. J. Wolfgang, C. L. Rosen, P. D'Eustachio, and J. L. Salzer. 1995. Cloning of neurotrimin defines a new subfamily of differentially expressed neural cell adhesion molecules. *J Neurosci* 15, no. 3 Pt 2: 2141-56.

Sundberg, C. and K. Rubin. 1996. Stimulation of beta1 integrins on fibroblasts induces PDGF independent tyrosine phosphorylation of PDGF beta-receptors. *J Cell Biol* 132, no. 4: 741-52.

Sundfeldt, K., Y. Piontkewitz, K. Ivarsson, O. Nilsson, P. Hellberg, M. Brannstrom, P. O. Janson, S. Enerback, and L. Hedin. 1997. E-cadherin expression in human epithelial ovarian cancer and normal ovary. *Int J Cancer* 74, no. 3: 275-80.

Taetle, R., M. Aickin, J. M. Yang, L. Panda, J. Emerson, D. Roe, L. Adair, F. Thompson, Y. Liu, L. Wisner, J. R. Davis, J. Trent, and D. S. Alberts. 1999. Chromosome abnormalities in ovarian adenocarcinoma: I. Nonrandom chromosome abnormalities from 244 cases. *Genes Chromosomes Cancer* 25, no. 3: 290-300.

Takeichi, M. 1995. Morphogenetic roles of classic cadherins. *Curr Opin Cell Biol* 7, no. 5: 619-27.

Tamura, M., J. Gu, E. H. Danen, T. Takino, S. Miyamoto, and K. M. Yamada. 1999. PTEN interactions with focal adhesion kinase and suppression of the extracellular matrix-dependent phosphatidylinositol 3-kinase/Akt cell survival pathway. *J Biol Chem* 274, no. 29: 20693-703.

Tanaka, Y., K. Mimori, T. Shiraishi, Y. Ohkura, K. Takubo, K. Mafune, G. F. Barnard, and M. Mori. 2000. alpha6 integrin expression in esophageal carcinoma. *Int J Oncol* 16, no. 4: 725-9.

Tashiro, H., K. Miyazaki, H. Okamura, A. Iwai, and M. Fukumoto. 1992. c-myc over-expression in human primary ovarian tumours: its relevance to tumour progression. *Int J Cancer* 50, no. 5: 828-33.

Thigpen, T., M. F. Brady, G. A. Omura, W. T. Creasman, W. P. McGuire, W. J. Hoskins, and S. Williams. 1993. Age as a prognostic factor in ovarian carcinoma. The Gynecologic Oncology Group experience. *Cancer* 71, no. 2 Suppl: 606-14.

Trimble, EL. 1999. Improved survival for women with ovarian cancer. *Proceedings of the Society for Gynecologic Oncology* : Abstract 136.

Truong, A. H. and Y. Ben-David. 2000. The role of Fli-1 in normal cell function and malignant transformation. *Oncogene* 19, no. 55: 6482-9.

Ueda, K., M. M. Cornwell, M. M. Gottesman, I. Pastan, I. B. Roninson, V. Ling, and J. R. Riordan. 1986. The mdr1 gene, responsible for multidrug-resistance, codes for P-glycoprotein. *Biochem Biophys Res Commun* 141, no. 3: 956-62.

van Dalen, A., J. Favier, A. Burges, U. Hasholzner, H. W. de Bruijn, D. Dobler-Girdziunaite, V. H. Dombi, D. Fink, M. Giai, P. McGing, A. Harlozinska, C. Kainz, J. Markowska, R. Molina, C. Sturgeon, A. Bowman, and R. Einarsson. 2000. Prognostic significance of CA 125 and TPS levels after 3 chemotherapy courses in ovarian cancer patients. *Gynecol Oncol* 79, no. 3: 444-50.

van Dam, P. A., I. B. Vergote, D. G. Lowe, J. V. Watson, P. van Damme, J. C. van der Auwera, and J. H. Shepherd. 1994. Expression of c-erbB-2, c-myc, and c-ras oncoproteins, insulin-like growth factor receptor I, and epidermal growth factor receptor in ovarian carcinoma. *J Clin Pathol* 47, no. 10: 914-9

.

Vergote, I., J. De Brabanter, A. Fyles, K. Bertelsen, N. Einhorn, P. Sevelda, M. E. Gore, J. Kaern, H. Verrelst, K. Sjovall, D. Timmerman, J. Vandewalle, M. Van Gramberen, and C. G. Trope. 2001. Prognostic importance of degree of differentiation and cyst rupture in stage I invasive epithelial ovarian carcinoma. *Lancet* 357, no. 9251: 176-82.

Verhoeven, D. T., R. A. Goldbohm, G. van Poppel, H. Verhagen, and P. A. van den Brandt. 1996. Epidemiological studies on brassica vegetables and cancer risk. *Cancer Epidemiol Biomarkers Prev* 5, no. 9: 733-48.

Vihinen, P., J. Nikkola, T. Vlaykova, M. Hahka-Kemppinen, L. Talve, J. Heino, and S. Pyrhonen. 2000. Prognostic value of beta1 integrin expression in metastatic melanoma [In Process Citation]. *Melanoma Res* 10, no. 3: 243-51.

Vincenti, M. P. and C. E. Brinckerhoff. 2001. Early response genes induced in chondrocytes stimulated with the inflammatory cytokine interleukin-1beta. *Arthritis Res* 3, no. 6: 381-8.

Walsh, G. M., F. A. Symon, A. L. Lazarovils, and A. J. Wardlaw. 1996. Integrin alpha 4 beta 7 mediates human eosinophil interaction with MAdCAM-1, VCAM-1 and fibronectin. *Immunology* 89, no. 1: 112-9.

Wei, M. H., F. Latif, S. Bader, V. Kashuba, J. Y. Chen, F. M. Duh, Y. Sekido, C. C. Lee, L. Geil, I. Kuzmin, E. Zabarovsky, G. Klein, B. Zbar, J. D. Minna, and M. I. Lerman. 1996. Construction of a 600-kilobase cosmid clone contig and generation of a transcriptional map surrounding the lung cancer tumor suppressor gene (TSG) locus on human chromosome 3p21.3: progress toward the isolation of a lung cancer TSG. *Cancer Res* 56, no. 7: 1487-92.

Welcsh, P. L. and M. C. King. 2001. BRCA1 and BRCA2 and the genetics of breast and ovarian cancer. *Hum Mol Genet* 10, no. 7: 705-13.

White, R. and P. O'Connell. 1991. Identification and characterization of the gene for neurofibromatosis type 1. *Curr Opin Genet Dev* 1, no. 1: 15-9.

Williams, EJ, B Mittal, FS Walsh, and P Doherty. 1995. FGF inhibits neurite outgrowth over monolayers of astrocytes and fibroblasts expressing transfected cell adhesion molecules. *J Cell Sci* 108, no. 11: 3523-3530.

Williams, E. J., J. Furness, F. S. Walsh, and P. Doherty. 1994. Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. *Neuron* 13, no. 3: 583-94.

Williams, E. J., F. S. Walsh, and P. Doherty. 1994. Tyrosine kinase inhibitors can differentially inhibit integrin-dependent and CAM-stimulated neurite outgrowth. *J Cell Biol* 124, no. 6: 1029-37.

Wilson. 1958. *Poultry Science* .

Wong, A. K., R. Pero, P. A. Ormonde, S. V. Tavtigian, and P. L. Bartel. 1997. RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2. *J Biol Chem* 272, no. 51: 31941-4.

Wong, C., R. E. Hempling, M. S. Piver, N. Natarajan, and C. J. Mettlin. 1999. Perineal talc exposure and subsequent epithelial ovarian cancer: a case-control study. *Obstet Gynecol* 93, no. 3: 372-6.

Wooster, R., G. Bignell, J. Lancaster, S. Swift, S. Seal, J. Mangion, N. Collins, S. Gregory, C. Gumbs, and G. Micklem. 1995. Identification of the breast cancer susceptibility gene BRCA2 [see comments] [published erratum appears in Nature 1996 Feb 22;379(6567):749]. *Nature* 378, no. 6559: 789-92.

Wu, R., D. C. Connolly, X. Ren, E. R. Fearon, and K. R. Cho. 1999. Somatic mutations of the PPP2R1B candidate tumor suppressor gene at chromosome 11q23 are infrequent in ovarian carcinomas. *Neoplasia* 1, no. 4: 311-4.

Xu, C. F. and E. Solomon. 1996. Mutations of the BRCA1 gene in human cancer. *Semin Cancer Biol* 7, no. 1: 33-40.

Xu, F., W. Xia, R. Z. Luo, H. Peng, S. Zhao, J. Dai, Y. Long, L. Zou, W. Le, J. Liu, A. F. Parlow, M. C. Hung, R. C. Bast, Jr., and Y. Yu. 2000. The human ARHI tumor suppressor gene inhibits lactation and growth in transgenic mice. *Cancer Res* 60, no. 17: 4913-20.

Yeh, E. T., T. Kamitani, and H. M. Chang. 1994. Biosynthesis and processing of the glycosylphosphatidylinositol anchor in mammalian cells. *Semin Immunol* 6, no. 2: 73-80.

Yoshiura, K., Y. Kanai, A. Ochiai, Y. Shimoyama, T. Sugimura, and S. Hirohashi. 1995. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proc Natl Acad Sci U S A* 92, no. 16: 7416-9.

Yu, Y., F. Xu, H. Peng, X. Fang, S. Zhao, Y. Li, B. Cuevas, W. L. Kuo, J. W. Gray, M. Siciliano, G. B. Mills, and R. C. Bast, Jr. 1999. NOEY2 (ARHI), an imprinted putative

tumor suppressor gene in ovarian and breast carcinomas. *Proc Natl Acad Sci U S A* 96, no. 1: 214-9.

Zeimet, A. G., E. Muller-Holzner, C. Marth, and G. Daxenbichler. 1994. Immunocytochemical versus biochemical receptor determination in normal and tumorous tissues of the female reproductive tract and the breast. *J Steroid Biochem Mol Biol* 49, no. 4-6: 365-72.

Zhu, X., M. Ohtsubo, R. M. Bohmer, J. M. Roberts, and R. K. Assoian. 1996. Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein. *J Cell Biol* 133, no. 2: 391-403.

Zisch, A. H., L. D'Alessandri, K. Amrein, B. Ranscht, K. H. Winterhalter, and L. Vaughan. 1995. The glypiated neuronal cell adhesion molecule contactin/F11 complexes with src-family protein tyrosine kinase Fyn. *Mol Cell Neurosci* 6, no. 3: 263-79.